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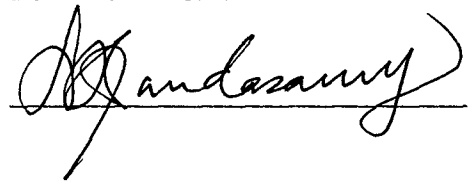

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These studies continue to focus on defining the mechanisms by which the E1A oncogene sensitizes formerly resistant human tumor cells to destruction by proapoptotic injuries. Comparison and contrast between human breast cancer cells and NIH-3T3 cells continues to be used to analyze genetic mechanisms and traits that are common among species and unique to human tumor cells. Other types of human tumor cells have been contrasted with breast cancer cells for this same reason. The original two tasks continue to provide the focus for this work. In the first task, cDNA array analysis of E1A-induced differential gene expression has progressed. Resulting studies have focused on assessment of the possible relevance of E1A modulation of osteopontin gene expression in detemining cellular susceptibility to proapoptotic injuries. In the second task, studies have focused on developing cell systems to test E1A-induce repression of antiapoptotic cellular mechanisms. Initial results show that E1A blockade of the cellular NF-kB defense against cytokine-induced apoptosis is one mechanism of sensitization to apoptosis. Tests of the generalization of this observation to breast cancer cells, among different breast cancer cell lines and among different types of human tumors are under way.

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INTRODUCTION:

These studies exploit our observation that expression of the E1A oncogene of human adenovirus types 5 converts formerly resistant normal and neoplastic cells into cells that become highly sensitive to proapoptotic injuries. The injuries tested include those inflicted by killer lymphocytes (both NK cells and cytotoxic T lymphocytes), cytokines such as TNF alpha (TNFα) and a variety of chemical agents, including chemotherapeutic drugs. The proposed experiments were designed to test translation of observations made during studies of mouse NIH-3T3 cells to human breast cancer cells. This comparative analysis has provided the basis for studies of the cellular pathways that can be modulated by E1A gene expression to mediate conversion of cells to the apoptosis-sensitive phenotype. Definition of these basic cellular mechanisms may lead to development of new strategies for enhancing the efficacy of both immunological and chemotherapeutic forms of antineoplastic therapy.

BODY:

Task one was to identify cellular genes whose expression is modulated by E1A to cause increased cellular sensitivity to proapoptotic injuries. In previous reports, it was noted that expression of the p53 tumor suppressor gene is not required for E1A-induced sensitivity to apoptotic injury. This observation was confirmed using both rodent and human tumor cells [1]. Notably, human breast cancer cells with loss of function mutations in p53 were sensitized by E1A expression to a variety of proapoptotic injuries, including both immune injuries (NK cells, TRAIL, anti-Fas antibody) and chemical injuries (e.g., etoposide). To confirm the lack of importance of p53 for this E1A-induced cellular phenotype, studies were done using the human osteosarcoma cell line, Saos-2, that is completely negative for p53 expression. These p53-negative cells were also rendered sensitive as a result of E1A expression to immune-mediated (NK cell) and chemically induced (etoposide, cytosine arabinoside, hydroxyurea, cisplatin) apoptotic injuries. This observation suggested that E1A-induced sensitivity to apoptosis was not restricted to human breast cancer cells but might be observed with a variety of human tumor cell types. To test this possibility, the studies were extended to human fibrosarcoma and melanoma cells [2]. Both cell types were rendered sensitive to immune-mediated (TRAIL-induced) apoptosis by E1A expression. These observations suggest that the process by which E1A induces this phenotypic change in human tumor cells is not restricted to a specific cell type or tissue type but may be generally applicable to many or all types of human tumors.

The complementary question to that of cell type specificity was whether there is oncogene specificity for induction of the apoptosis-sensitive phenotype in tumor cells. To test this, cells were compared following stable transfection with either the adenoviral E1A oncogene or the E7 oncogene from human papilloma virus type 18. The question was whether both E1A and E7 would induce cellular sensitivity to proapoptotic injuries. Initial studies have been completed contrasting NK cell-induced killing of mouse fibrosarcoma cells expressing either of these two oncogenes [3]. The results indicate that E1A, but not E7, induces sensitivity to NK cell-induced apoptosis. Similar studies have

also been completed using human breast carcinoma cells. The results are the same, since E7-expressing breast cancer cells are no more susceptible to killer cell-induced apoptosis than nontransfected cells, whereas E1A-expression in the same cells resulted in high level sensitization to killer cell-induced apoptosis. Therefore, despite the fact that E1A and E7 share several other oncogene activities, including induction of cellular immortalization, changes in cellular transcriptional control and related cell cycle regulation, these oncogenes differ markedly in their abilities to induce cellular sensitivity to proapoptotic injuries. In addition to defining the E1A specificity of apoptosis sensitization, these data provide a basis for comparative genetic analyses of E1A and E7 that might lead to definition of the oncogene region(s) that are required to trigger the apoptotic cellular phenotype.

Studies described in the initial proposal identified the existence of E1A-related differences in cellular gene expression in NIH-3T3 cells. It was postulated that one or more of these differences in gene expression might explain the change in the apoptotic response in cells expressing E1A. Progress has been made in comparisons of gene expression between E1A-negative and E1A-positive cells using cDNA array technology. As predicted by previous differential display experiments, there are numerous E1A-related gene expression changes detected by this assay. These changes are being reviewed and assessed for possible implication in control of the apoptotic phenotype. A specific question that was posed during this analysis was whether the initially observed difference in osteopontin gene expression detected with NIH-3T3 cells would also be observed with human breast cancer cells. This comparison has been completed using the Affymetrix system, and the results have been confirmed. E1A expression in breast cancer cells represses osteopontin gene expression by approximately tenfold. This E1A-induced repression might be interesting in the context of the sensitivity of tumor cells to apoptotic injury because of the apparent relationship between the effects of osteopontin binding to its integrin receptor (alpha v beta 3) and changes in the cellular response to chemical stimulation [e.g., 4]. This observation about differential gene expression in E1A expressing provides the basis for a series of experiments to test the hypothesis that E1A-induced repression of osteopontin is causally linked to the change in apoptosis sensitivity of neoplastic cells. These studies are in progress.

Task two was to assess the level in the cellular apoptosis response at which E1A expression mediates conversion of cells from apoptosis-resistant to apoptosis-sensitive. As previously reported, we have excluded the role of the Bcl-2 family member, E1B 19 kD proteins in blocking immune-mediated apoptosis [1]. We had preliminary data that the NF-kappa B activation response to proapoptotic injury was compromise in E1A-positive cells. This observation has been confirmed and is being translated from studies in NIH-3T3 cells to studies in human breast cancer cells. The status of the results is that E1A expression has been observed to block TNF-induced NF-kappa B activation and thereby to sensitize cells to TNF induced apoptosis. The data to date indicate that the mechanism of this E1A blockade of NF-kappa B does not involve reduction in cytokine signaling, nuclear translocation of the transcription factor or transcription factor binding to its enhancer. The results point to a repressive interaction at the level of the transcription complex. Several human breast cancer cell systems are being tested for

translation of these data to human tumor cells and to determine whether this observation can be generalized to different cell lines. This experimental system provides a focused area of investigation to pursue for definition of a molecular mechanism of the sensitization to apoptosis.

Discussion. Progress has been made on this project and several fronts. We have observed that, in contrast to rodent cells, p53-mutant or p53-negative human tumor cells can be sensitized by E1A expression to apoptosis triggered by both immune-mediated and chemotherapy drug-induced injuries. This observation is important for analysis of the relevance of the E1A Control mechanism, considering the frequency of p53 mutations in human breast cancer. These data suggest that caution is warranted when translating observations about apoptosis control mechanisms from mouse to human cell systems. Our studies of the cellular pathways through which E1A controls the response to injury have been complemented by evaluations of differential gene expression in E1A-positive versus E1A-negative cells. The goal is to use the gene expression studies to both test possible mechanisms suggested by cytotoxicity assays and to suggest new directions for experimentation. This triple approach of evaluation of E1A-induced changes in cellular response to proapoptotic injuries, E1A-related alterations in the cellular antiapoptotic response and E1A-induced changes in a spectrum of genes using c DNA array technology is consistent with the originally proposed scope of work and provides the best opportunity for progress in the studies of the control mechanisms that determine the outcome of tumor cell injury.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified the difference between NIH-3T3 cells and human breast cancer cells in the p53 tumor suppressor gene expression requirement for E1A-induced sensitization to immune mediated and chemically induced apoptosis.

- Extended the spectrum of human tumor cells studied to include breast cancer cells, fibrosarcoma cells and melanoma cells, revealing that E1A-induced sensitization to apoptotic injury is not restricted cells of one tumor or tissue type.

- Confirmed that E1A represses osteopontin gene expression in human breast cancer cells as well as in NIH-3T3 cells and have initiated studies of the relevance of osteopontin repression for E1A-induced sensitization to proapoptotic injuries.

- Identified the NF-kappa B activation response as a target of E1A activity that provides one explanation for sensitization to cytokine-induced apoptosis and have initiated studies to translate this observation to human breast cancer cells.

REPORTABLE OUTCOMES:

1. Cook, JL, Routes, BA, Walker, T, Colvin, KL, and Routes, JM. E1A oncogene induction of susceptibility to killing by cytolytic lymphocytes through target cell sensitization to apoptotic injury. Experimental Cell Research **251**: 414-423, 1999.

2. Cook, JL, Routes, BA, Leu, C, Walker, TA, and Colvin, KL. E1A oncogene-induced cellular sensitization to immune-mediated apoptosis is independent of p53 and resistant to blockade by E1B 19 kD protein. Experimental Cell Research **252**: 199-210, 1999.
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5. Routes, JM, Ryan, S, Li, H, Steinke, J, and Cook, JL Dissimilar immunogenicities of human papillomavirus E7 and adenovirus E1A proteins influence primary tumor development. Virology **277**(1): 48-57., 2000.
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9. Cook, JL, Walker, TA, and Radke, JR E1A Repression of NF-kB p65/RelA-dependent transcription sensitizes cells to TNF-induced apoptosis independently of E1A protein binding to p300 or TBP. Manuscript in revision, 2001.

CONCLUSIONS:

The results from the studies indicate that E1A-induced sensitization of neoplastic cells to both immune-mediated and chemically induced apoptosis is a common property of rodent cells and human breast cancer cells. The results do show, however, that there are species-related differences in the pathways through which this oncogene-induced phenotype is controlled. Creation of a breast cancer cells system in which to study E1A-induced apoptosis sensitivity provides a basis for continued analysis of the level in the apoptosis cascade through which this phenotypic conversion is controlled. The results to date suggest that studies of cytokine-induced activation of the NF-kappa B defense and of the possible role of osteopontin repression in changing cellular sensitivity to injury may be productive. Since NF-kappa B activation is a well-known factor in the apoptosis cascade and osteopontin is over expressed in a majority of breast cancer cells, these avenues of investigation may be productive and are being pursued.

"So what?" -- The long-term goal of this project has been consistent throughout the period of funding -- to use the adenoviral E1A oncogene as a tool to identify cellular pathways and molecular mechanisms that can be used to convert chemotherapy- and immunotherapy-resistant breast cancer cells into cells that are more sensitive to these

therapeutic interventions. The results show important similarities and differences between mouse cell models and human breast cancer cells that provide useful information for interpretation of studies from these two types of cell systems. The identification of specific cellular targets of E1A activity provide clear directions for future experiments to define the molecular triggers that can be used to sensitize tumor cells to therapeutic interventions.

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LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

1. James L. Cook MD
2. Kelly L Colvin
3. Vivian Zheng
4. Yong Kang

Appendix

Experimental Cell Research **251,** 414–423 (1999)
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E1A Oncogene Induction of Cellular Susceptibility to Killing by Cytolytic Lymphocytes Through Target Cell Sensitization to Apoptotic Injury

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E1A oncogene expression increases mammalian cell susceptibility to lysis by cytolytic lymphocytes (CLs) at a stage in this intercellular interaction that is independent of cell surface recognition events. Since CLs can induce either apoptotic or necrotic cell death, we asked whether E1A sensitization to injury-induced apoptosis is sufficient to explain E1A-induced cytolytic susceptibility. Mouse, rat, hamster, and human cells that were rendered cytolytic susceptible by E1A were also sensitized to CL-induced and chemically induced apoptosis. In contrast, E1A-positive cells were no more susceptible to injury-induced necrosis than E1A-negative cells. Similar to induction of cytolytic susceptibility and in contrast to other E1A activities, cellular sensitization to chemically induced apoptosis depended on high-level E1A oncoprotein expression. Loss of both cytolytic susceptibility and sensitization to chemically induced apoptosis was coselected during *in vivo* selection of E1A-positive sarcoma cells for increased tumorigenicity. Furthermore, E1A mutant proteins that cannot bind the cellular transcriptional coactivator, p300, and that fail to induce cytolytic susceptibility also failed to sensitize cells to injury-induced apoptosis. These data indicate that E1A induces susceptibility to killer cell-induced lysis through sensitization of cells to injury-induced apoptosis.

Key Words: **adenovirus; E1A; oncogene; cytolytic lymphocyte; beauvericin; apoptosis; NIH-3T3.**

INTRODUCTION

In addition to its roles in controlling viral gene expression and the cell cycle, the E1A oncogene of human adenovirus (Ad) types 2 and 5 also actively induces cells to become highly susceptible to lysis by several components of the antitumor immune response, including different types of cytotoxic lymphocytes (CLs), activated macrophages, and tumor necrosis factor α (TNFα) [15–17, 19, 45, 55]. This E1A-induced cellular phenotype has been termed "cytolytic susceptibility." We and others proposed that E1A-induced cytolytic susceptibility explains the lack of tumorigenicity of cells transformed by these nononcogenic Ad serotypes in immunocompetent animals [8, 10, 19, 44, 45, 49, 50, 55].

We reported that the mechanism by which E1A induces the cytolytic susceptible phenotype involves a stage in the interaction between CLs and their E1A-expressing target cells that follows, and is independent of, interactions between killer cell or cytokine ligands and target cell receptors—a "postrecognition" stage in cellular injury [18]. These observations suggest that E1A induces cytolytic susceptibility by causing a qualitative change in the cellular response to CL-induced injury.

Most reports indicate that CLs kill their target cells by inducing apoptosis through two mechanisms: one caused by the joint actions of CL granule-associated perforin and granzymes and the other resulting from CL surface Fas-ligand crosslinking of Fas antigen on target cells (reviewed in [2, 5]). However, there is also evidence suggesting that CL-induced apoptosis may not explain all killing activity and that CLs can also cause a necrotic cell death response in at least some types of target cells [20, 51, 54]. Therefore, it was not possible, using only assays of CL-induced injury, to determine whether E1A induction of cytolytic susceptibility is caused by E1A-induced cellular sensitivity to apoptosis or necrosis or to both cell death responses.

In the studies presented in this report, fibroblastic cells stably transfected with the E1A oncogene and expressing the E1A oncoprotein were used to test the relationship between E1A induction of cytolytic susceptibility and E1A sensitization to apoptotic injury.

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The results of studies of the E1A expression level dependence of these two cellular phenotypes and of their linkage during *in vivo* selection and E1A mutational analysis suggest that the mechanism by which E1A induces cytolytic susceptibility involves E1A sensitization to killer cell-induced apoptosis.

MATERIALS AND METHODS

Cells and cell lines. NIH-3T3 cells were obtained from the American Type Culture Collection (ATCC). Several E1A-expressing cell lines derived from NIH-3T3 are represented in the figures as follows. The terms NIH-3T3+, 3T3-E1A, and E1A 289R Hi (see [1–3, 5] and Table 1) refer to the cell line, 13–2, which expresses a high level of the 289R oncoprotein encoded by the E1A 13S cDNA [16]. E1A 289R Lo (Fig. 2A) refers to a similarly transfected clone, 13–3, that expresses a low level of E1A 289R [16]. E1A 243R Hi and E1A 243 Lo (Fig. 2A) refer to two clones, MT12SA and 12–3, that express either a high or a low level of E1A 12S cDNA-encoded protein, 243R, respectively [16]. C3.11 (Fig. 2B) is a cell line established using the Lac Switch inducible mammalian expression system (Stratagene, La Jolla, CA). For this purpose, the E1A gene was synthesized by polymerase chain reaction and replaced the CAT gene in the pOPRS-VICAT plasmid. This plasmid was cotransfected with the p3′SS plasmid into NIH-3T3 cells, and transfected clones were selected in hygromycin and geneticin. The C3.11 subclone was selected for its expression of high-level E1A on induction with IPTG. 3T3-PSdl and 3T3-NCdl cells (Fig. 5) are NIH-3T3 clones established following transfection of NIH-3T3 cells with the E1A mutant genes E1A-PSdl and E1A-NCdl, as described [9]. E1A-PSdl deletes all of E1A conserved region 1 (CR1) but does not affect expression of the N-terminal 22 amino acids of E1A [36]. The E1A-NCdl mutation deletes amino acids 61 through 85 but does not affect expression of either the E1A N terminus or E1A CR1 [37]. The E1A-PSdl mutation eliminates the E1A gene regions required for oncoprotein binding to the cellular p300 transcriptional coactivator and retinoblastoma (Rb)-family member proteins [3, 9, 36]. The E1A oncoprotein encoded by E1A-NCdl mutant gene retains the ability to bind p300 or Rb-family proteins [9, 56].

H4+ (Fig. 1) is an E1A-transfected H4 cell line, P2AHT2A [25]. The H4-derived cell line, H4-RG2-Clone 2, has been described [46]. This cell line was created by transfecting H4 cells with the E1A plasmid, 12S.RG2, which contains an E1A gene point mutation that results in an arginine-to-glycine change at the second amino acid in the amino terminus of the E1A oncoprotein [57]. H4-RG2-Clone 2 expresses the mutant E1A protein at a high level [46]. The E1A-RG2 oncoprotein fails to bind cellular p300, but continues to bind Rb-family proteins [56].

RN12+ (Fig. 1; formal name RN12-1Agpt-A) is a cell line that expresses E1A at a high level and that was derived by transfection of RN12 cells with p1Agpt [24]. BHK21+ (Fig. 1) is an E1A-expressing clone, D5, of BHK-21 cells [55].

Hamster embryo cells (HECs) were prepared and used as described [11]. ATL-1 and ATL-2 and cell lines were derived from tumors developing in adult hamsters after serial *in vivo* transplantation of the Ad2-transformed HEC line AdHE3 [14].

E1A expression levels in these cell lines were compared by immunoblotting using the monoclonal antibody M73 [26]. High-level E1A expression denotes an oncoprotein expression level that is comparable to that detected in virally transformed or virally infected rodent cells. Cells expressing E1A at low levels have been previously reported and compared with high level expressers [16].

All of these E1A-positive cell lines grow well *in vitro* and, of particular relevance for this study, do not spontaneously undergo apoptosis *in vitro*. Furthermore, where tested (BHK-21-D5, Ad2HE3, and P2AHT2A), the E1A-positive cells are able to form tumors in immunodeficient animals [11, 19, 55], suggesting that these cells also do not spontaneously undergo apoptosis *in vivo*.

Assays of injury-induced apoptosis and necrosis. Each beauvericin, gramicidin, and etoposide (VP16) lot (Sigma, St. Louis, MO) was titered against E1A-negative NIH-3T3 cells and the E1A-positive NIH-3T3 cell line 13–2, across a broad range of concentrations to determine optimal culture conditions for detecting E1A-specific sensitization to chemical injury. Initial studies of beauvericin-induced apoptosis were done using the apoptosis-sensitive cell line EL-4 as a control for comparison to E1A-positive cells. Assays of CL-induced apoptosis were done using the cytotoxic T lymphocyte (CTL) clone 4.1 and the lectin PHA-P, as described [18]. Apoptotic and necrotic cell death induced by these agents was confirmed by microscopic examination of nuclear morphological changes of injured cells [22] and was quantitated using 6-h ^{51}Cr release assays as described [55] and as validated in these studies. For cell morphology studies, cells stained with DNA-binding dyes were scored based on the characteristics of aberrant chromatin organization as described [22]. The significance of the differences observed was estimated using Student's t test with JMP software from the SAS Institute. Low-molecular-weight DNA ("fragmented nuclear DNA") release from injured cells undergoing apoptosis [22] was used as a third means to quantitate apoptosis (Table 1).

RESULTS

E1A Induction of Cytolytic Susceptibility Correlates with Sensitization to Apoptotic Injury

To begin to define the mechanism by which E1A sensitizes cells to killing by CLs, we initially determined whether there was a correlation between E1A sensitization to apoptosis induced by both CLs and chemically induced injuries. The prediction was that different types of E1A-positive cytolytic susceptible cells should simultaneously exhibit traits of CL-induced apoptosis and acquire sensitivity to apoptosis induced by unrelated proapoptotic, chemical stimuli. Mouse, rat, hamster, and human cells that had been rendered highly susceptible to CL-induced lysis as a result of E1A transfection [16, 18, 45, 55] were tested for sensitivity to apoptosis induced by the potassium ionophore beauvericin (Fig. 1). This proapoptotic agent was chosen because its mechanism of induction of apoptosis is distinct from the perforin/granzyme and Fas-ligand activities by which CLs trigger apoptosis. Beauvericin induces release of intracellular calcium stores and appears to activate one or more cellular endonucleases that cause apoptosis in susceptible cells [41]. Therefore, it was possible to ask the question whether E1A expression induced sensitivity to diverse proapoptotic stimuli.

E1A-positive, CL-susceptible cells from all four species were highly sensitive to beauvericin-induced cell death, whereas the respective E1A-negative control cells were resistant (Fig. 1). To confirm that the cell death was apoptotic in nature, these E1A-positive cells were examined by fluorescence microscopy for beauvericin-induced cell shrinkage and nuclear chromatin condensation, which are diagnostic of apoptosis [22]. In

all cases, E1A-positive cells showed apoptotic nuclear changes within 30 min of beauvericin treatment, whereas the cellular and nuclear morphologies of E1A-negative cells were unaffected by beauvericin. Beauvericin did not cause necrotic cell death of either E1A-positive or E1A-negative cells. Less than 5% of cells in all treated preparations showed the diffuse nuclear staining with ethidium bromide that is diagnostic of necrosis [22]. Therefore, the E1A-induced sensitization of cells to beauvericin was apoptosis specific. Apoptosis-specific cell death was also observed with E1A-positive cells injured by CLs. Fluorescence microscopy of E1A-positive NIH-3T3 cells that had been injured by cocultivation with the 4.1 clone of CL (at a 50:1 CL: target cell ratio) for 6 h revealed apoptosis-specific nuclear fragmentation and condensation in the majority of target cells. Less than 5% of these CL-injured target cells in repeated assays showed cellular morphologies indicative of necrotic death (data not shown).

Another characteristic of cells undergoing apoptosis is degradation of their DNA into low-molecular-weight fragments as a result of internucleosomal chromatin cleavage [22]. This apoptotic response can be assessed qualitatively by DNA "laddering" patterns detected during agarose gel electrophoresis and quantitatively by measuring the percentage of DNA that is released as low-molecular-weight fragments [22]. DNA laddering was observed with beauvericin-treated, E1A-positive cells from all four species tested but not with DNA extracted from E1A-negative cells (not shown). DNA fragmentation was quantitated for E1A-positive and E1A-negative NIH-3T3 cells and compared with patterns of beauvericin-induced apoptotic nuclear morphology and cell death, as quantitated by ^{51}Cr release (Table 1). Both CLs and beauvericin caused a significant increase in cellular DNA fragmentation of E1A-positive, but not E1A-negative, cells. Beauvericin effects on E1A-positive cells were preceded by the nuclear morphology characteristic of apoptosis. The observations that E1A-positive cells from different species exhibited apoptotic, but not necrotic, cell death responses validated the subsequent use of the radiorelease assay to quantitate injury-induced apoptosis. This strong positive correlation between E1A-induced susceptibility to lysis by CLs and E1A-induced sensitization to CL- and beauvericin-induced apoptosis suggested that target cell lysis and apoptosis are two manifestations of the same E1A activity.

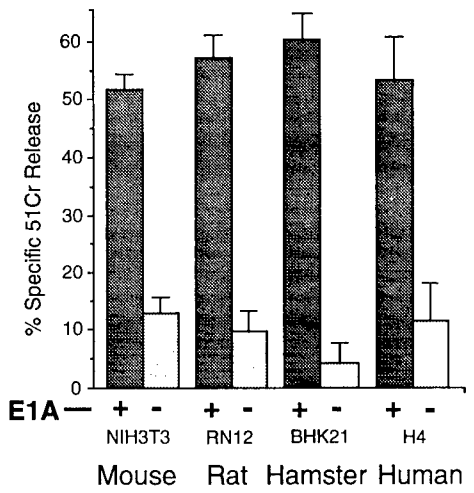

FIG. 1. Increased beauvericin sensitivity of NK-susceptible E1A-expressing cell lines from four different species. E1A-positive (dark bars) and E1A-negative mouse, rat, hamster, and human fibroblastic cells were tested for sensitivity to beauvericin-induced killing in 6-h ^{51}Cr-release assays. Bars represent the mean ± SEM results of three (RN12, BHK-21, H4) to eight (NIH-3T3) experiments using beauvericin at a final concentration of 10 μM (mouse, rat, and human cells) or 13 μM (hamster cells). E1A-positive cells of all four types were significantly more sensitive to beauvericin-induced cell death than the respective E1A-negative cells ($P < 0.05$).

TABLE 1

Evidence for Apoptotic Death of E1A-Positive NIH-3T3 Cells Injured by Cytolytic Lymphocytes or Beauvericin

Injury	Target Cell	
	NIH-3T3	3T3-E1A
Cytolytic lymphocytes		
Fragmented nuclear DNA (2 h)	33 ± 2%	70 ± 3%
^{51}Cr release (6 h)	19 ± 5%	58 ± 5%
Beauvericin		
Apoptotic nuclei (30 min)	10 ± 2%	85 ± 5%
Fragmented nuclear DNA (2 h)	13 ± 5%	47 ± 8%
^{51}Cr release (6 h)	16 ± 2%	64 ± 3%

Note. Enumeration of apoptotic cells and quantitation of fragmented nuclear DNA release were done as described a [22]. Quantitation of cell death was estimated by radiolabel release as described [55]. Each set of comparison data represents the mean ± SEM results of at least three experiments. For each index of measurement, the E1A-positive cells (3T3-E1A) were significantly more sensitive to apoptotic injury than the E1A-negative cells (NIH-3T3) ($P < 0.05$).

E1A Sensitization to Apoptosis, Like E1A-Induced Cytolytic Susceptibility, Depends on High-Level Oncoprotein Expression

Our previous studies showed that E1A-induced susceptibility to lysis by natural killer cells and activated macrophages is dependent on the relatively high levels of expression of E1A oncoproteins similar to those found during Ad infection of permissive cells or Ad-induced cell transformation [16, 19]. As a further test of the correlation between the requirements for E1A-induced cytolytic susceptibility and E1A sensitization to apoptotic injury, we used two different types of cells to evaluate the level of E1A oncoprotein expression required for beauvericin-induced apoptosis. NIH-3T3

cells stably transfected with cDNAs from either of the two major E1A mRNAs (13S or 12S) and clonally selected for expression of either high or low levels of the E1A oncoproteins (289R and 243R, respectively) were tested for sensitivity to beauvericin (Fig. 2A). Transfected cells expressing high levels of both E1A proteins were shown previously to be highly susceptible to lysis by natural killer lymphocytes (NK cells) and activated macrophages, whereas the transfectants expressing low levels of these E1A proteins remained resistant to lysis by both types of killer cells [16]. An identical pattern of sensitivity was detected to beauvericin-induced apoptosis. Cells expressing high-level E1A 289R or 243R proteins were sensitive to beauvericin. In contrast, cells expressing low levels of E1A proteins were no more susceptible than E1A-negative NIH-3T3 cells (Fig. 2A).

We next tested whether clonal differences between these lines, other than E1A-induced effects, could explain the patterns of beauvericin sensitivity observed. For this purpose, a cell line (C3.11) was created in which all cells in the population could be induced to express the E1A 289R oncoprotein when treated with IPTG. C3.11 cells expressed a barely detectable level of E1A 289R protein by Western blotting in the absence of IPTG induction (Fig. 2B). When treated with IPTG, essentially all cells in the population (>95% by E1A-specific immunofluorescence) expressed E1A 289R protein at a level as high as that detected in the E1A 289R-Hi expresser cell line represented in Fig. 2A. Uninduced C3.11 cells were resistant to lysis by different types of killer lymphocytes (not shown), indicating that the trace level of E1A expression in the uninduced state was insufficient to change their inherent cytolytic resistance. C3.11 cells became highly susceptible to CL-induced lysis during the first 24 h after IPTG induction when the cells expressed high levels of E1A 289R protein (not shown).

The same pattern of dependence on E1A oncoprotein expression level was observed when a time–response study was done to assess sensitivity to chemically induced apoptosis in C3.11 cells treated with beauvericin (Fig. 2B). In the uninduced state, cells expressing trace levels of E1A 289R were no more sensitive to beauvericin-induced apoptosis than E1A-negative NIH-3T3 cells (Fig. 2B). C3.11 cells became increasingly sensitive to injury-induced apoptosis through 36 h of E1A induction (Fig. 2B, right three bars). Comparison of the E1A expression and apoptosis sensitization data revealed that there may also be a time dependence of E1A sensitization apoptosis. E1A expression level did not increase after 18 h of IPTG stimulation (Fig. 2B). However, E1A-expressing cells exhibited increasing sensitivity to beauvericin-induced apoptosis throughout 36 h of testing (Fig. 2B). Beauvericin-injured, E1A-positive C3.11 cells exhibited diagnostic apoptotic changes in nuclear morphology at each time point that were identical to those seen with cells stably expressing high levels of E1A 289R.

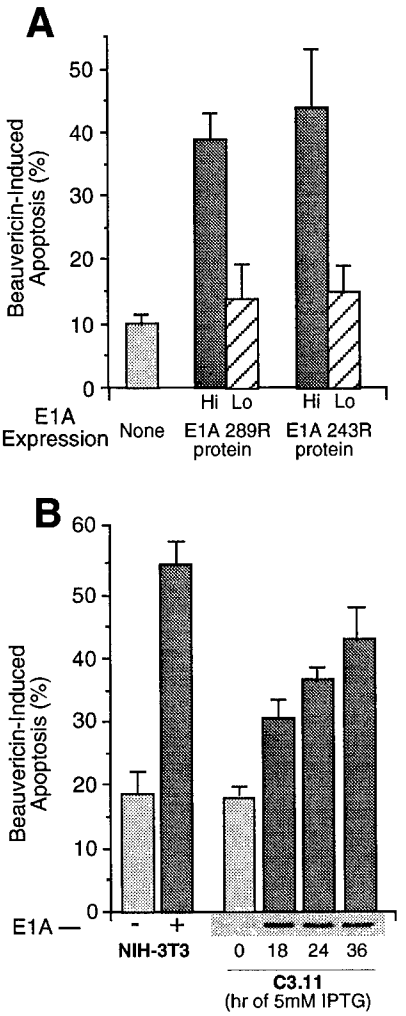

FIG. 2. E1A expression level dependence of cellular sensitivity to apoptotic injury. NIH-3T3 cells expressing E1A proteins at high or low levels (as indicated) following either (A) stable transfection with E1A 13S (289R protein) or 12S (243R protein) cDNAs, respectively [16], or (B) IPTG induction of E1A 13S cDNA (289R protein) expression (C3.11 cells) were tested for sensitivity to apoptosis induced by beauvericin at a final concentration of 13 μM. Bars in (A) represent the mean ± SEM results of three to six experiments. Cells expressing high levels of either E1A 289R or E1A 243R were significantly more sensitive to beauvericin-induced apoptosis than either E1A-negative NIH-3T3 cells or cells expressing the respective E1A proteins at low levels ($P < 0.05$). Bars in (B) represent the mean ± SEM results of four assays in which the beauvericin sensitivities of C3.11 cells were tested before and at the indicated times after IPTG induction of E1A 289R expression. E1A-negative NIH-3T3 cells and the stably transfected, E1A-positive cell line 13–2 were used as beauvericin-resistant and beauvericin-sensitive controls, respectively. IPTG-induced C3.11 cells were significantly more sensitive to beauvericin-induced apoptosis than untreated control cells at 24 and 36 h after IPTG treatment ($P < 0.05$ by ANOVA using Dunnett's comparison).

These data on C3.11 cells, coupled with those on cells stably expressing high or low levels of E1A shown in Fig. 2A, indicated that E1A sensitization to injury-induced apoptosis was dependent on a relatively high threshold level of E1A oncoprotein expression. The finding that both E1A-induced cytolytic susceptibility and E1A sensitization to chemically induced apoptosis were dependent on high-level oncoprotein expression suggested that these two E1A-induced cellular phenotypes were manifestations of the same E1A activity.

E1A Does Not Sensitize Cells to Injury-Induced Necrotic Cell Death

The DNA degradation patterns and nuclear morphology changes observed with beauvericin-injured, E1A-positive cells were consistent with apoptosis. However, it has been reported with other types of cells that beauvericin can also cause cellular mitochondrial changes similar to those observed in cells undergoing necrotic cell death [41]. Furthermore, it has been suggested that CLs can cause cellular necrosis, as well as apoptosis in some types of target cells [31], although most reported evidence indicates that apoptosis is the major form of CL-induced cell death [23]. To test whether any of the detected sensitization to cell death that is induced by E1A can be explained by increased cellular susceptibility to necrosis, E1A-positive and E1A-negative cells were compared for sensitivity to the potassium ionophore gramicidin, an agent that causes necrotic cell death (R. Duke, personal communication).

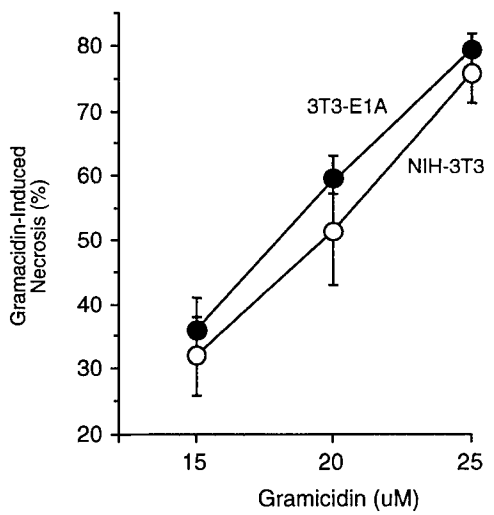

FIG. 3. Lack of E1A sensitization of NIH-3T3 cells to necrotic cell death. E1A-negative and E1A-positive (13–2) cells were compared for sensitivity to chemically induced necrosis at the indicated concentrations of gramicidin. Points represent the mean ± SEM results of three experiments. E1A-positive cells were not more sensitive to gramidicin-induced necrosis than E1A-negative cells ($P > 0.10$).

In these experiments, E1A-positive and E1A-negative NIH-3T3 cells were equally sensitive to gramicidin-induced necrosis (Fig. 3). The absence of an E1A-sensitizing effect to necrotic cell death was also observed with matched E1A-positive and E1A-negative hamster, rat, and human cells treated with gramicidin (not shown). Morphological studies of cells treated with gramicidin confirmed that the dying cells exhibited the diffuse nuclear staining with ethidium bromide that is characteristic of necrotic cell death [22] and lacked the cell shrinkage and nuclear chromatin condensation seen with apoptotic cells. Therefore, high-level E1A expression sensitizes cells to apoptosis induced by both CLs and beauvericin, but E1A does not sensitize cells to gramicidin-induced necrotic cell death. These results are compatible with our reported observation that E1A-expressing cells are no more sensitive than E1A-negative cells to killing by antibody plus complement [18], another form of cellular injury that induces necrotic death.

Loss of E1A Sensitization to Injury-Induced Apoptosis is Coselected with Loss of Cytolytic Susceptibility during Neoplastic Progression of Ad2-Transformed Rodent Sarcoma Cells

We have described an Ad2-transformed hamster sarcoma model in which weakly tumorigenic, E1A-positive cells were adapted by serial *in vivo* passage to become highly tumorigenic in immunocompetent animals [14]. These "adapted" tumor cell lines, ATL-1 and ATL-2, retained identical patterns of adenoviral gene integration into the cellular genome and continued to express E1A oncoproteins at the same high levels seen with their weakly tumorigenic, parental sarcoma cell line, Ad2HE3. In comparison with parental cells, however, the ATL lines lost cytolytic susceptibility as evidenced by reduced killing by NK cells and tumor cell-activated macrophages compared with Ad2HE3 cells [14].

To further test the correlation between E1A-induced cytolytic susceptibility and E1A sensitization to apoptotic injury, the two CL-resistant ATL cell lines were compared for sensitivity to beauvericin-induced apoptosis with their CL-susceptible parental cell, Ad2HE3, and with the nontransformed HECs from which Ad2HE3 was derived (Fig. 4). The results showed that the CL-resistant ATL lines had also lost susceptibility to beauvericin-induced apoptosis relative to CL-susceptible Ad2HE3 cells and that the ATL lines were no more susceptible to beauvericin than CL-resistant HECs. Therefore, these E1A-positive ATL cells that were selected *in vivo* for loss of cytolytic susceptibility were coselected for loss of sensitivity to chemically induced apoptosis. This linkage between the loss of these two cellular phenotypes strengthened the case

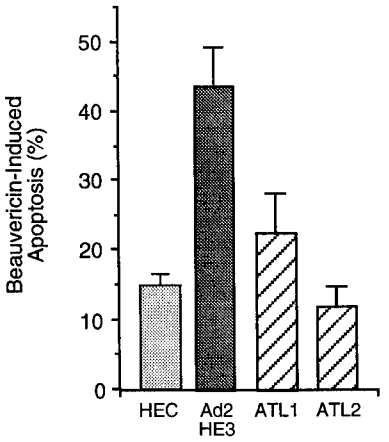

FIG. 4. Loss of apoptosis sensitivity of E1A-positive fibrosarcoma cells following *in vivo* selection for loss of susceptibility to NK killing during serial tumor transplantation. Tumor lines, ATL-1 and ATL-2, derived from *in vivo* passage of the NK-susceptible hamster cell line Ad2HE3 were compared with parental cells for susceptibility to apoptosis induced by beauvericin at a final concentration of 10 μM. Primary HECs (from which Ad2HE3 were derived) were used as apoptosis-resistant control cells. Ad2HE3 cells are highly susceptible to lysis by hamster NK cells, whereas ATL-1 cells, ATL-2 cells, and HECs cells are NK resistant [14]. Bars represent the mean ± SEM results of five experiments. ATL-1 cells, ATL-2 cells, and HECs were significantly less susceptible to beauvericin-induced apoptosis than Ad2HE3 cells ($P < 0.05$).

for a causal relationship between E1A sensitization to apoptotic injury and E1A-induced cytolytic susceptibility.

E1A Mutation That Eliminates Induction of Cytolytic Susceptibility Also Eliminates E1A-Induced Sensitization to Apoptotic Injury

We reported that first-exon mutations involving either the N terminus or conserved region 1 (CR1) of the E1A gene abrogated E1A-induced susceptibility of hamster cells to lysis by NK cells, despite continued high-level oncoprotein expression following either viral infection or stable transfection [9]. These E1A gene regions are required for oncoprotein binding to the cellular transcriptional coactivator protein, p300. E1A first-exon mutations that did not prevent E1A binding to p300 did not block E1A-induced cytolytic susceptibility. These results suggested the importance of E1A–p300 binding interactions for induction of cytolytic susceptibility.

E1A mutational analysis was used to test the correlation between E1A-induced sensitization to apoptotic injury and induction of susceptibility to lysis by killer cells. NIH-3T3 cells expressing high levels of two different mutant E1A oncoproteins that differ in their binding to p300 were compared with cells expressing wild-type E1A protein for sensitivity to apoptosis induced by killer cells and beauvericin (Fig. 5A). 3T3-PSdl cells, which express an E1A mutant protein, E1A-PSdl, lacking the CR1 binding domain required for p300 binding [9] remained highly resistant to both killer cell- and beauvericin-induced apoptosis. 3T3-PSdl cells were as resistant to injuries as nontransfected NIH-3T3 cells. In contrast, 3T3-NCdl cells, which express an E1A mutant protein, E1A-NCdl, that continues to bind p300 as well as wild-type E1A protein [9] were as sensitive to both proapoptotic injuries as target cells expressing wild-type E1A. Cells expressing either wild-type E1A or E1A-NCdl proteins exhibited nuclear morphologies and low-molecular-weight DNA fragment release patterns diagnostic of apoptosis, whereas cells expressing E1A-PSdl protein were indistinguishable from E1A-negative cells in these assays. The observation that the E1A-PSdl mutation abrogated E1A-induced cellular sensitization to apoptotic injury suggested the importance of E1A–p300 interactions in this E1A activity. However, since the PSdl mutation can also affect E1A binding to Rb-family proteins, these data did not exclude a role for E1A–Rb interactions in sensitization to injury-induced apoptosis.

To refine the analysis of the requirement for E1A–p300 binding interactions for cellular sensitization to proapoptotic injuries, human H4 fibrosarcoma cells expressing either wild-type (wt) E1A oncoprotein or an E1A mutant protein encoded by E1A 12S.RG2 were compared for susceptibility to injury-induced apoptosis (Fig. 5B). The E1A mutant gene 12S.RG2 contains a point mutation that causes an arginine-to-glycine switch at the second amino acid of the E1A oncoprotein and results in loss of p300 binding [57] but does not affect Rb-family protein binding by E1A (our unpublished data). The H4 cell line RG2-Clone 2 was selected for these experiments because it expresses a high level of the mutant E1A RG2 protein. Three types of proapoptotic injuries—human NK cells (Hu NK), beauvericin (BR), and etoposide (Etop)—were used to test the effect of the RG2 mutation on the ability of E1A to sensitize human cells to apoptosis. Etoposide is a topoisomerase II inhibitor that triggers apoptosis in susceptible cells [32]. Fibrosarcoma cells expressing wt E1A were susceptible to apoptosis induced by all three injuries (Fig. 5B). In contrast, H4-RG2-Clone 2 cells were as resistant to all three injuries as parental E1A-negative fibrosarcoma cells. Subsequent coimmunoprecipitation studies confirmed that the E1A–RG2 mutant protein expressed in these cells failed to form complexes with the p300 transcriptional coactivator but did form complexes with Rb-family member proteins (data not shown). These data confirm the observations using the larger E1A deletion mutation, PSdl, by showing that an E1A mutation that eliminates oncoprotein binding to p300 also eliminates the E1A activity that sensitizes cells to injury-induced apoptosis and suggest

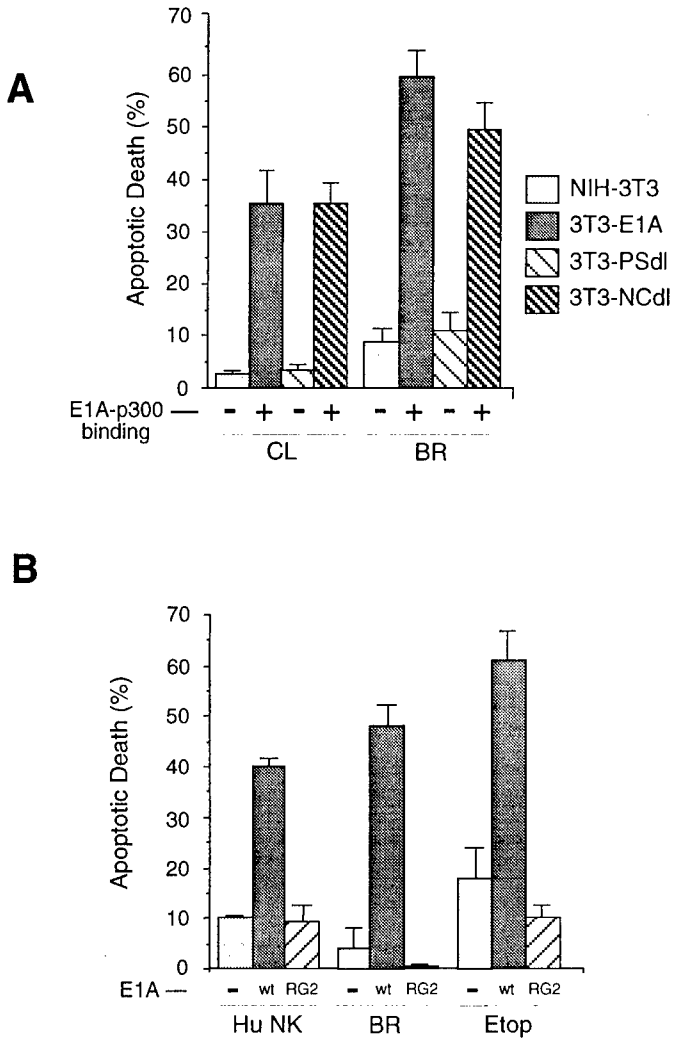

FIG. 5. Failure of E1A mutant oncoproteins that cannot bind the cellular p300 transcriptional coactivator to sensitize mouse (A) and human (B) cells to apoptotic injuries. (A) NIH-3T3 transfected clones, 3T3-PSdl and 3T3-NCdl, were compared with E1A-negative (NIH-3T3) and E1A-positive (3T3-E1A) controls for sensitivity to apoptosis induced by either cytotoxic lymphocytes (CLs; 4.1 cells at a killer cell-to-target cell ratio of 6:1) or beauvericin (final concentration = 10 μM). Bars represent the mean ± SEM results of three experiments with each type of injury. Binding of wild-type or mutant E1A proteins to p300 as detected by immunoprecipitation with E1A-specific antibody followed by immunoblotting with anti-p300 antibody as described [9] is indicated. 3T3-PSdl cells (no E1A–p300 binding detected) were significantly less susceptible to apoptosis induced by both types of injuries than cells expressing either wild-type E1A or E1A–NCdl ($P < 0.05$), both of which showed E1A–p300 binding. In contrast, 3T3-NCdl cells were equally susceptible to CLs and beauvericin compared with 3T3-E1A cells. (B) Human H4 fibrosarcoma cells transfected with the E1A point mutation gene 12S.RG2 were compared with nontransfected parental cells and cells transfected with the wild-type (wt) E1A gene for susceptibility to three types of proapoptotic injuries. Hu NK = human natural killer cell assay results, using a 200:1 blood mononuclear cell-to-target cell ratio and a cocultivation period of 6 h. BR = beauvericin at final concentration of 11 μM in 6-h apoptosis assays. Etop = etoposide at a final concentration of 100 μg/ml in 18-h apoptosis assays. Bars represent the mean ± SEM results of two (BR) or three (Hu NK and Etop) experiments with each type of injury. H4-RG2-CL39 cells were significantly less susceptible to injury-induced apoptosis triggered by all three types of injury than cells expressing wt E1A ($P < 0.05$), but RG2-CL-39 cells were not significantly more susceptible to proapoptotic injury than parental, E1A-negative H4 cells.

that E1A binding to cellular Rb-family proteins is insufficient to sensitize cells to apoptotic injury.

DISCUSSION

These studies provide several types of evidence that indicate a cause-and-effect relationship between E1A sensitization to injury-induced apoptosis and E1A induction of cellular susceptibility to lysis by killer cells. First, the association between these two E1A-induced cellular phenotypes was found to be a general property of cells from four different species (Fig. 1). Second, there were similar dose–response relationships between E1A oncoprotein expression and sensitization to injury-induced apoptosis (Fig. 2) and E1A oncoprotein expression and induction of cytolytic susceptibility [16, 19]. In both cases, high-level expression similar to that observed during viral infection or virus-induced neoplastic transformation of cells is required for the E1A-related cellular phenotype. In contrast, other E1A activities, including transcriptional activation [59], transcriptional repression [53], cellular immortalization [1], and support of adenoviral replication [27], require only low levels of E1A protein expression. Third, cell selection *in vivo* for loss of E1A-induced cytolytic susceptibility coselects for loss of E1A sensitization to injury-induced apoptosis (Fig. 4). Fourth, E1A gene mutations that caused the oncoprotein to lose the ability to induce cytolytic susceptibility also caused loss of E1A-induced sensitization to injury-induced apoptosis (Fig. 5). Fifth, E1A sensitization to injury-induced cell death was specific for apoptosis, since E1A did not sensitize cells to injury-induced necrosis (Fig. 3). This specificity of E1A sensitization for apoptotic injury is compatible with our reported observation that E1A does not sensitize cells to necrotic cell death induced by treatment with antibody plus complement [18]. All of these observations support the conclusion that E1A sensitization to apoptotic injury and E1A-induced susceptibility of target cells to lysis by killer cells are manifestations of the same E1A oncoprotein activity.

CLs can induce both apoptotic and necrotic cell death responses, depending on the target cell tested [20, 28, 31, 54, 60]. The data in this report show that the major mechanism by which E1A renders cells more susceptible to CL-induced lysis is E1A sensitization to apoptosis, not necrosis. E1A expression did not sensitize cells to either CL-induced or chemically (gramicidin) in-

duced necrosis. These results suggest the conclusion that pronecrotic activities of CLs are not important in the *in vivo* rejection of E1A-expressing tumor cells by immunocompetent animals.

There was a strong correlation between increased levels of E1A oncoprotein expression and sensitization to killer cell-induced apoptosis in these studies (Fig. 2). These results provide an explanation for the E1A expression level dependence of expresser cell susceptibility to killing by NK cells, activated macrophages, and TNFα. For example, we reported that adenovirus-induced susceptibility of infected hamster fibroblasts to killing by activated macrophages increases with increasing multiplicity of viral infection and the concomitant increased expression of viral early proteins, including E1A [12]. We also observed that high-level, but not low-level, E1A oncoprotein expression causes increased susceptibility of BHK-21 cells to NK cell killing [19] and of NIH-3T3 cells to killing by NK cells, activated macrophages, and recombinant TNFα [16]. All three of these types of immune-mediated injuries can induce apoptosis in susceptible target cells. These correlations lead us to suggest that the loss of tumorigenicity of E1A-expressing BHK-21 cells in immunocompetent (but not immunodeficient) animals represents the *in vivo* consequence of E1A sensitization of tumor cells to immune-mediated apoptosis [19, 55].

The reasons for the time delay between reaching maximum E1A expression in the inducible NIH-3T3 cell line, C3.11, and detection of maximum sensitivity to apoptotic injury (Fig. 2B) are unknown. It is possible that, as the E1A oncoprotein is expressed, it must alter the function of one or more targeted cellular molecules, such as the p300 transcriptional coactivator, before complete sensitization to apoptotic injury can be achieved. This would suggest that a certain threshold of titration of the targeted molecule(s) by E1A must occur before cellular sensitization to apoptotic injury is complete. We have reported a correlation between E1A interactions with the p300 transcriptional coactivator and induction of susceptibility of Ad-infected and E1A-transfected cells to killing by natural killer lymphocytes [9]. Most reports indicate that E1A–p300 interactions result in repression of p300-dependent cellular transcription. It is possible, therefore, that E1A must titrate a p300-dependent transcriptional response to sensitize cells to injury-induced apoptosis.

There is evidence that E1A itself can also initiate apoptosis, either directly or indirectly, without prior cellular injury. When E1A is expressed during either viral infection or initiation of oncogene-induced cellular immortalization, it triggers apoptosis without a requirement for any subsequent cellular injury [4, 6, 21, 29, 30, 38–40, 42, 43, 47, 48, 58]. Most of these reports suggest that this direct induction of cellular apoptosis by E1A is the consequence of its stimulation of unscheduled cellular DNA synthesis that, in turn, triggers a p53-dependent apoptosis pathway. In studies from one laboratory, it was shown that E1A can promote apoptosis in virally infected cells indirectly through transcriptional activation of the adenoviral E4 gene which then triggers p53-independent apoptosis [35, 52]. In another report, E1A was shown to cause apoptosis of virally infected cells independently of both E4 and p53 expression [7]. The requirement for injury of cells stably expressing E1A to undergo apoptosis distinguishes the E1A activity described in this report from studies in which E1A expression itself causes cellular apoptosis during viral infection. The E1A-transfected cell lines studied here, and others we have tested, are viable during long-term tissue culture passage and, where evaluated, have induced tumors in immunodeficient animals [8, 9, 13, 55]. Therefore, in these selected, E1A-transfected cell lines, there are no apparent adverse effects of E1A oncoprotein expression until the cells are exposed to proapoptotic injuries. The viability of these E1A-expressing cells does not require coexpression of other collaborating genes, such as E1B [43].

Other reports of E1A-induced sensitization of cells to nonimmune, proapoptotic injuries have demonstrated a requirement for p53 expression for this E1A effect [33, 34]. This p53 requirement is also similar to that reported in most of the aforementioned studies of E1A-related apoptosis occurring during viral infection or the establishment of the immortalized state. Our unpublished results indicate that E1A-induced cytolytic susceptibility and E1A sensitization to immune-mediated apoptosis are independent of p53 expression (J. L. Cook, B. A. Routes, T. W. Walker, and K. L. Colvin, submitted for publication). Whether all of these relationships between cellular sensitization to apoptotic injury and E1A expression during infection, immortalization, and stable transfection using different assays of apoptosis are different manifestations of the same E1A activity and whether there are different E1A-controlled cellular pathways that affect the cellular apoptotic response are questions that require further, direct comparison studies.

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E1A Oncogene-Induced Cellular Sensitization to Immune-Mediated Apoptosis Is Independent of p53 and Resistant to Blockade by E1B 19 kDa Protein

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E1A oncogene expression sensitizes mammalian cells to apoptosis triggered by cytolytic lymphocytes (CL) [16]. Most studies suggest that E1A-induced apoptosis involves a p53-dependent cellular pathway that is blocked by the E1B 19 kDa gene product. In this study, the roles of p53 and E1B 19 kDa were tested for E1A sensitization to CL-induced apoptosis in contrast with apoptosis triggered by TNF α or chemical injuries. E1A sensitization to immune-mediated (CL- or TNF-induced) apoptosis was independent of p53 expression and was resistant to blockade by E1B 19 kDa protein in mouse and hamster cells. In contrast, the p53 requirement for chemically induced apoptosis of E1A-sensitized cells varied with the agent used to treat cells. Apoptosis induced by diverse chemical agents (hygromycin, beauvericin, etoposide, H_2O_2) was blocked by E1B 19 kDa expression. Therefore, both the p53-dependence and the E1B 19 kDa blockade of E1A-induced cellular sensitization to apoptotic injury depend on the type of proapoptotic injury tested. These data suggest that the mechanisms by which E1A sensitizes tumor cells to immune-mediated apoptosis and to rejection by immunocompetent animals do not require cellular expression of wild-type p53 and can function independently of the Bcl-2-like, antiapoptotic mechanisms of E1B 19 kDa. © 1999 Academic Press

Key Words: **adenovirus; E1A; cytolytic lymphocyte; tumor necrosis factor alpha; apoptosis; p53; E1B 19 kDa.**

INTRODUCTION

Expression of the E1A oncogene of human adenovirus (Ad) types 2 and 5 renders mammalian cells from several species susceptible to lysis by components of the host cellular immune response, including both major types of cytolytic lymphocytes (CL)—natural killer (NK) cells and cytotoxic T lymphocytes (CTL)—activated macrophages, and tumor necrosis factor α (TNF) [1, 8, 23, 25, 32, 44, 71, 80]. We and others have postulated that this E1A-induced cytolytic susceptibility explains the inability of Ad-transformed cells and E1A-expressing tumor cells to form tumors in immunocompetent animals [14, 17, 68, 71, 75, 79, 90]. This hypothesis is supported by our recent observation that elimination of innate and specific cellular immunity *in vivo* also progressively eliminates host rejection of E1A-positive sarcoma cells (J. M. Routes, S. Ryan, H. Li, J. Steinke, and J. L. Cook, submitted for publication). The molecular mechanisms by which E1A sensitizes cells to these immune-mediated injuries are unknown, however.

We have reported that E1A sensitizes cells to killing by both types of CL-induced cellular injuries—degranulation-dependent and Fas-dependent injuries [24]. Fas-dependent injury by CL is specific for apoptosis [reviewed in 2]. CL-induced, degranulation-dependent injury mediated by the collaborative interaction of perforin and granzymes can cause either apoptosis or necrotic cell death, apparently depending on the target cell type and assay conditions [27, 82, 89]. Our recent studies indicate that degranulation-dependent injury by CL causes apoptotic, and not necrotic, cell death in E1A-expressing rodent fibroblasts [16]. We were, therefore, interested in testing the cellular requirements for CL-induced apoptosis of E1A-positive cells.

p53 antioncogene expression is required in other circumstances in which E1A induces apoptosis, including viral infection and oncogene-induced cellular immortalization [9, 29, 53, 62, 66, 67, 73, 93]. Testing of the role of p53 in E1A-induced sensitivity to CL-induced apoptosis has not been reported. However, the following indirect evidence suggested that p53 could be involved. It has been proposed that E1A-induced, p53-dependent apoptosis caused by other stimuli requires E1A binding to the p300 protein [66], which is a tran-

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scriptional coactivator for p53 [52, 78, 83]. Furthermore, E1A-induced cellular sensitization to killing by CL maps to the p300 binding regions of E1A [15].

E1A-related, p53-dependent apoptosis can be blocked by coexpression of the Ad E1B 19 kDa oncogene [4, 29, 67, 72]. E1B might protect virally infected cells from undergoing premature, E1A-induced apoptosis. The E1B protective effect might also explain the increased efficiency of cellular immortalization of rodent cells cotransfected with E1A and E1B compared to cells transfected with E1A alone. E1B 19 kDa blockade of apoptosis triggered by exogenous cellular injuries is variable. E1B 19 kDa can block TNF-induced apoptosis [10, 37, 42, 93], but this can be cell-type- or species-specific [48, 87]. This is similar to the cell-system-specific, TNF blocking effect of Bcl-2 [69], the antiapoptotic cellular gene with which E1B shares limited sequence homology and functional activity. E1B 19 kDa and Bcl-2 blockade of Fas-induced apoptosis is also variable [10, 12, 60, 77, 84]. Our previous reports showed no E1B blockade of E1A-induced susceptibility to CL-induced killing of Ad-infected, Ad-transformed, or oncogene-transfected tumor cells [25, 71, 90], suggesting that E1B 19 kDa might not effectively block E1A-induced sensitization to CL-induced apoptosis.

In these studies, p53-negative and p53-positive mouse cells were compared for E1A-induced sensitization to immune-mediated or chemically triggered apoptosis. Mouse and hamster cells expressing E1A alone or coexpressing E1A plus E1B 19 kDa were contrasted to test the efficacy of E1B blockade of E1A sensitization to apoptosis. The results showed that expression of wild-type p53 was not required for E1A to sensitize cells to immune-mediated apoptosis, but that some types of chemically induced injuries required p53 to trigger apoptosis of E1A-sensitized cells. E1A sensitization to immune-mediated apoptosis was resistant to E1B 19 kDa blockade, whereas E1B blocked apoptosis induced by several different types of chemical injuries. The implications of these observations for the mechanisms by which E1A sensitizes cells to immune-mediated apoptosis and to rejection by immunocompetent animals are discussed.

MATERIALS AND METHODS

Cell lines. NIH-3T3 cells were obtained from the ATCC. The E1A-positive clone, 13-2 (represented in the figures as 3T3-E1A), has been described [23]. 13-2 expresses high-level E1A 289R oncoprotein encoded by the E1A 13S cDNA. (10)3 cells are 3T3-like, mouse fibroblasts with a mutation that creates a stop codon in the p53 coding sequence and have no detectable p53 protein [41]. (10)3-E1A (Figs. 3, 6, and 7 and Table 1), refers to a (10)3 subclone, A22, that expresses E1A at a comparable level to 13-2 by immunoblotting with the monoclonal antibody, M73 [40]. A22 was created by transfection of (10)3 with the plasmid, p1A-pac, and selection in puromycin. The E1A-positive, Saos-2 human osteosarcoma cell line was provided by S. Frisch and expresses E1A 243R protein (encoded by the E1A 12S cDNA) at a level comparable to the E1A in 13-2 cells.

Three cell lines were used for studies testing E1B 19 kDa effects on sensitivity to apoptotic injury (Fig. 4)—NIH-3T3 (E1A- and E1B-negative), F411 (E1A-positive, E1B-negative), and E1PB9 (E1A-positive, E1B 19 kDa-positive). F411 was created by transfecting NIH-3T3 with p1A-pac and selecting E1A-expressing clones in puromycin. E1PB9 was created by cotransfecting NIH-3T3 with the plasmids p5*Xho*I-C [3] and pPUR (Clontech, Palo Alto, CA) and selecting E1A-expressing clones in puromycin. A second selection was done to identify E1A-positive clones that expressed E1A 19 kDa protein, as assessed by immunoblotting using the antibody, 1G11 (Oncogene Sciences, San Diego, CA). The BHK-21 lines (Fig. 7C) have been described [26, 90]. The cell line represented as BHK-E1A+E1B is BHK-D5 that expresses E1A and E1B 19 kDa oncoproteins at high levels. The cell line represented as BHK-E1A is BHK-B2 which expresses only E1A oncoproteins at the same high level as BHK-D5 cells [26].

Assays of injury-induced apoptosis. CL-induced apoptosis of mouse target cells was assessed using the mouse CTL clone, 4.1 [46], in lectin-dependent cellular cytotoxicity assays as described [24]. 4.1 kills by degranulation-dependent mechanisms in calcium-rich medium and cannot mediate Fas-dependent killing without prior activation of Fas-ligand expression [24]. Degranulation-dependent, Fas-independent killing by 4.1 was used here. CL-induced apoptosis of human osteosarcoma cells was assessed using human NK cells as described [25, 71]. Recombinant mouse TNF α (Genzyme, Cambridge, MA), hygromycin (Cal Biochem, La Jolla, CA), beauvericin (Sigma, St. Louis, MO), etoposide (Sigma) and H_2O_2 (Bergen Brunswig, Orange, CA) were tested in dose–response studies of NIH-3T3 and E1A-positive, 13-2 to define optimal concentrations for detecting E1A-specific sensitization to apoptotic injury (Table 1). Apoptosis induced by these agents was assessed by evaluating the nuclear morphology of injured cells [33]. All of these injuries induced nuclear fragmentation and chromatin condensation in E1A-positive target cells. In contrast, less than 5% of cells injured with any agent showed the diffuse nuclear staining with ethidium bromide that indicates cellular necrosis. Low-molecular-weight DNA release from injured cells [33] was used as a second test to define apoptotic cell death. Our studies have validated the use of [^{51}Cr] release (6 h for CL, NK, and beauvericin and 18 h for TNF, hygromycin, etoposide, and H_2O_2) to quantitate apoptotic death in assays such as these where injury-induced necrosis has been excluded [16]. The significance of the differences in apoptotic cell death was estimated by analysis of variance using the JMP program from SAS Institute.

RESULTS

E1A-induced sensitization of target cells to immune-mediated apoptosis does not require p53 expression. To test the p53 requirement for immune-mediated apoptosis, assays of degranulation-dependent killing by CL were used to compare apoptotic injury of E1A-expressing, p53-negative, and p53-positive mouse fibroblasts. E1A expression caused p53-negative, (10)3 cells to become as sensitive to CL-induced apoptosis as E1A-positive, p53-positive, NIH-3T3 cells, across a 10-fold range of CL to target cell ratios (Fig. 1). Therefore, p53 expression was not required for either the mechanism by which CL injured cells or the mechanism by which E1A sensitized cells to apoptotic injury. Identical results were obtained with the p53-negative, human Saos-2 cells [31], in which E1A expression caused increased susceptibility to apoptosis induced by human NK cells (Fig. 2).

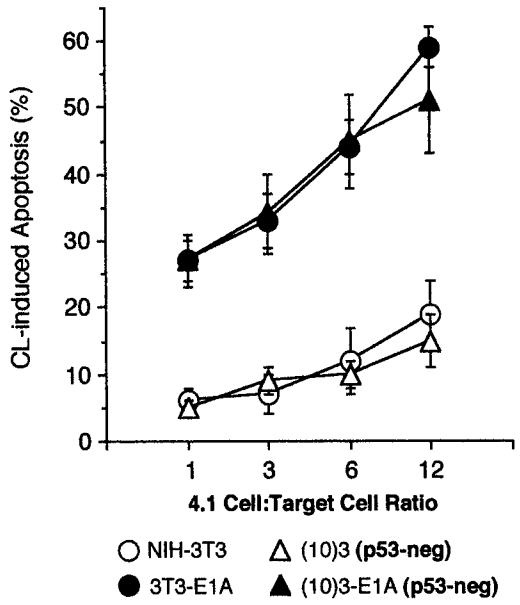


FIG. 1. E1A-induced sensitization of mouse fibroblasts to apoptosis induced by cytolytic lymphocytes (CL) is not affected by the absence of cellular, p53 expression. p53-negative, (10)3 cells were contrasted with p53-positive, NIH-3T3 cells for the ability to exhibit E1A sensitization to CL-induced apoptosis. The mouse CTL clone, 4.1, was used as the source of CL-induced injury in these experiments. Points represent the mean ± SEM results of three experiments in which CL-induced apoptotic cell killing was quantitated at CL:target cell ratios from 1:1 to 12:1. E1A-positive, p53-negative cells were equally susceptible to CL-induced apoptosis as E1A-positive, p53-positive cells ($P > 0.10$). E1A-negative cells of both types were resistant to CL-induced apoptosis. E1A-positive cells of both types were significantly more susceptible to CL-induced apoptosis than were the respective E1A-negative cells ($P < 0.05$).

In addition to cytotoxic T lymphocytes and NK cells (the main types of CL *in vivo*), the activated macrophage is another major component of the host cellular immune response to neoplastic cells. The main cytotoxic mediator used by activated macrophages is the cytokine, TNFα, which is responsible for the majority of macrophage cytolytic activity against E1A-positive cells [23]. TNF can induce p53-dependent cellular responses [43, 94], but can also trigger apoptosis in the absence of p53 [47, 95]. To test the p53 requirement for E1A sensitization to TNF-induced apoptosis, the same set of p53-negative and p53-positive mouse cells used for CL apoptosis assays was tested for apoptotic sensitivity in TNF dose–response experiments (Fig. 3). E1A expression sensitized p53-negative and p53-positive mouse fibroblasts equally to TNF-induced apoptosis. Therefore, p53 expression was not required for the E1A activity that sensitizes cells to injury by either CL (Figs. 1 and 2) or TNF (Fig. 3).

The p53 requirement for E1A-induced cellular sensitization to apoptosis is dependent on the proapoptotic injury tested. In contrast to these results of immune-mediated cellular injury, it is reported that E1A sensitization of mouse fibroblasts to chemotherapeutic agents and ionizing irradiation is strictly p53-dependent [55]. This suggested the possibility that the apoptotic sensitivity of E1A-positive cells could be a function of a p53 requirement for *triggering* apoptosis by certain injuries, and not a reflection of whether E1A had *sensitized* the cells to injury-induced apoptosis per se. To test this, different chemical injuries (hygromycin, beauvericin, etoposide, and H_2O_2) were contrasted with CL- and TNF-induced injuries for induction of apoptosis in p53-positive and p53-negative mouse cells expressing E1A (Table 1). E1A-positive, p53-positive cells were susceptible to apoptosis induced by all six types of injuries. In contrast, E1A-positive, p53-negative cells were susceptible to apoptosis induced by CL, TNF, and hygromycin, but were highly resistant to apoptosis induced by beauvericin, etoposide, and H_2O_2. The hygromycin result showed that some chemical injuries do not require p53 expression to trigger apoptosis in E1A-sensitized cells. The beauvericin, etoposide, and H_2O_2 results showed that other chemical injuries can require p53 expression to trigger apoptosis in E1A-sensitized cells. More important as a general observation was that the apparent sensitivity of E1A-expressing cells to apoptosis is completely dependent on the type of proapoptotic injury chosen for the experiment.

E1B 19 kDa protein cannot prevent E1A sensitization to immune-mediated apoptosis but can block apoptosis

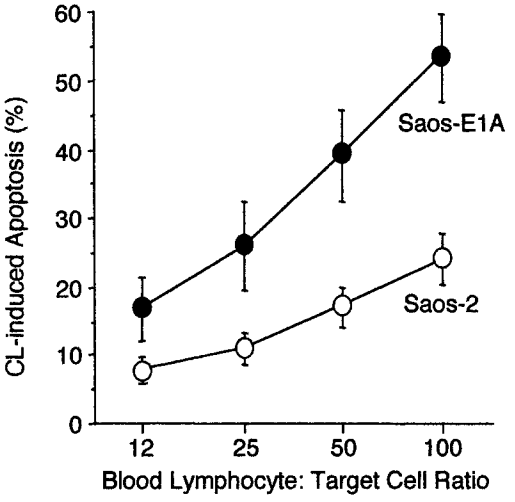


FIG. 2. E1A sensitizes p53-negative, human tumor cells to NK cell-induced apoptosis. Human Saos-2, osteosarcoma cells stably expressing E1A oncoprotein but lacking the p53 gene were compared with E1A-negative control cells for sensitivity to killer-cell-induced apoptosis. Human NK cells were used as the source of CL-induced injury. Points represent the mean ± SEM results of six experiments in which apoptotic killing by human NK cells was quantitated at blood lymphocyte:target cell ratios from 12:1 to 100:1. E1A-positive, Saos-2 cells were significantly more susceptible to NK-cell-induced apoptosis than E1A-negative cells at blood lymphocyte:target cell ratios of 50:1 and 100:1 ($P < 0.05$).

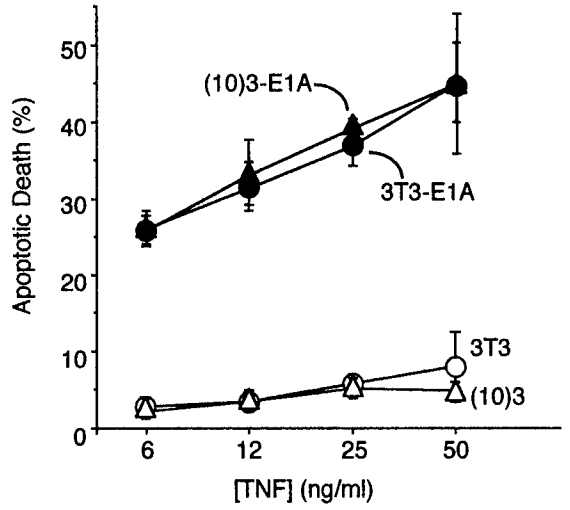


FIG. 3. E1A sensitization to TNF-induced apoptosis is independent of p53 expression. The same set of NIH-3T3 mouse target cells tested in the experiments represented in Fig. 1 was tested for sensitivity to apoptosis induced by recombinant, mouse TNFα at the indicated TNF concentrations. Points represent the mean ± SEM results of three experiments. E1A expression induced both p53-negative, (10)3 cells and p53-positive, NIH-3T3 cells to become significantly more susceptible to TNF-induced apoptosis than the respective E1A-negative control cells ($P < 0.05$). There was no significant difference in the TNF sensitivity of E1A-positive, p53-negative cells contrasted with E1A-positive, p53-positive cells ($P > 0.10$).

induced by chemical injuries in NIH-3T3 cells. E1B 19 kDa is an adenoviral gene-encoded protein that is a member of a family of viral and cellular proteins, which inhibit apoptosis, with Bcl-2 being the prototype [reviewed in 92]. E1B 19 kDa, like Bcl-2, can block some types of proapoptotic injuries, but not others [12, 60, 64, 69]. We reported that E1B expression does not prevent E1A induction of cellular susceptibility of rodent or human cells to lysis by NK cells, CTL, or activated macrophages [19, 22, 24–26, 50, 71, 90].

Studies were done to test the prediction from those observations that E1B 19 kDa would not block CL- or TNF-induced apoptosis of E1A-positive cells and to contrast the E1B effects on immune-mediated apoptosis with its effects against chemically triggered apoptosis of E1A-positive cells. p53-positive, NIH-3T3 cell clones were used that expressed either E1A alone or both E1A and E1B 19 kDa genes. The levels of E1A and E1B 19 kDa oncoprotein expression in these clones as detected by immunoblot with oncoprotein-specific monoclonal antibodies were comparable to the oncoprotein levels commonly detected in oncogene-transformed mouse, rat, hamster, and human cell lines (data not shown). The apoptotic sensitivities of these cells were contrasted using six injuries—CL, TNF, hygromycin, beauvericin, etoposide, and H_2O_2—under conditions that had been optimized for causing apoptosis of cells expressing only E1A (Fig. 4). E1B 19 kDa had no significant inhibitory effect on apoptosis induced by either CL or TNF in these assays. In contrast, E1B 19 kDa coexpression blocked E1A-induced sensitization to apoptosis induced by hygromycin, beauvericin, etoposide, and H_2O_2 down to levels that were not significantly different from those observed with E1A-negative, NIH-3T3 cells. The ability of E1B 19 kDa to block E1A sensitization to apoptosis was not linked to the p53-dependence of the injury (Table 1), since hygromycin (a p53-independent injury) and all three p53-dependent injuries (beauvericin, etoposide, and H_2O_2) were blocked, but CL and TNF (both p53-independent injuries) were not.

E1B 19 kDa protein expression does not block E1A sensitization immune-mediated apoptosis in either mouse fibroblasts or hamster sarcoma cells. The ability to detect blockade of injury-induced apoptosis by E1B 19 kDa and the functionally similar antiapoptotic molecule, Bcl-2, has varied depending on the injury and cell system tested [10, 37, 42, 48, 69, 87, 93]. The data in Fig. 4 showed that, using relatively high effector to target cell ratios (i.e., 50 CL to 1 target cell) at which CL-induced apoptosis of E1A-sensitized cells is maximized, E1B 19 kDa coexpression did not block

TABLE 1

Injury Specificity of the p53-Dependence of E1A Sensitization to Apoptosis

	Target cells			
Cell characteristics	NIH-3T3	3T3-E1A	(10)3	(10)3-E1A
p53	+	+	−	−
E1A	−	+	−	+
Injury	%Apoptosis			
CL (E:T = 6:1)	12 ± 5	44 ± 4	10 ± 2	45 ± 7
TNFα (25 ng/ml)	6 ± 1	42 ± 8	4 ± 3	44 ± 5
Hygromycin (800 ug/ml)	4 ± 2	56 ± 9	18 ± 2	70 ± 8
Beauvericin (12 uM)	1 ± 1	60 ± 9	1 ± 1	2 ± 1
Etoposide (25 uM)	9 ± 4	55 ± 8	7 ± 2	4 ± 1
Hydrogen peroxide (0.7 mM)	6 ± 1	51 ± 3	1 ± 3	9 ± 3

Note. The apoptosis sensitizing effect of E1A expression on p53-negative, (10)3 cells was compared with the E1A effect on p53-positive, NIH-3T3 cells using a variety of proapoptotic injuries. The percentage of apoptotic cell death was quantitated by radiolabel release. The apoptotic nature of cell death was confirmed by enumeration of apoptotic nuclei. Each set of data represents the mean ± SEM results of at least three experiments. E1A-positive, NIH-3T3 cells were significantly more sensitive than E1A-negative NIH-3T3 cells to apoptosis induced by all six injuries ($P < 0.05$). E1A-positive, (10)3 cells were significantly more sensitive than E1A-negative (10)3 cells to cytolytic lymphocytes (CL), TNFα, and hygromycin ($P < 0.05$). The differences in the sensitivities of E1A-positive and E1A-negative (10)3 cells to apoptosis induced by beauvericin, etoposide, and hydrogen peroxide were not significant ($P > 0.10$).

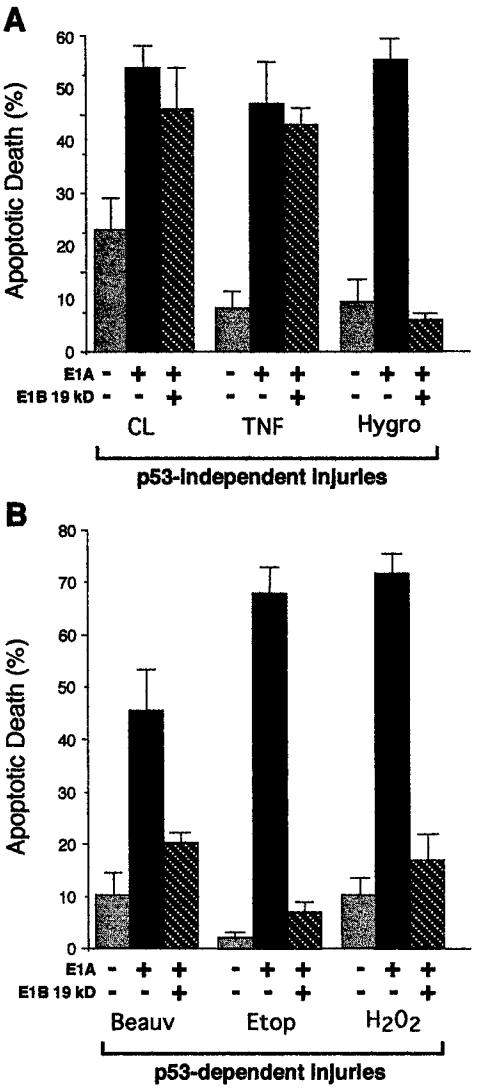


FIG. 4. E1B 19 kDa protein expression does not block E1A sensitization to immune-mediated apoptosis, but blocks chemically induced apoptosis of E1A-positive cells. NIH-3T3 cells expressing either E1A alone (E1A+ = 3T3-E1A cells) or both the E1A and the E1B 19 kDa oncoproteins (E1A+, E1B 19 kDa+ = E1PB9 cells) were tested under conditions which had been optimized for triggering apoptosis of E1A-positive cells by the six different injuries indicated. The three injuries represented in A (CL, TNF, and hygromycin) induced apoptosis independently of target cell p53 expression, whereas the three injuries represented in B (beauvericin, etoposide, and H_2O_2) required p53 expression to induce apoptosis (Table 1). Bars represent the mean ± SEM results of three or four experiments in which each injury was tested against all three cell types. E1A-positive cells were significantly more sensitive to apoptotic death caused by all six injuries than E1A-negative cells ($P < 0.05$). E1B 19 kDa protein coexpression had no inhibitory effect on the sensitivity of E1A-positive cells injured with the two immune mediators of apoptosis, CL, and TNFα ($P > 0.10$). In contrast, E1B 19 kDa coexpression caused a significant reduction in the ability of all four chemical injuries to induce apoptosis in E1A-positive cells ($P < 0.05$), resulting in a reduced level of apoptosis sensitivity that was not significantly greater than that exhibited by E1A-negative, NIH-3T3 cells ($P > 0.10$).

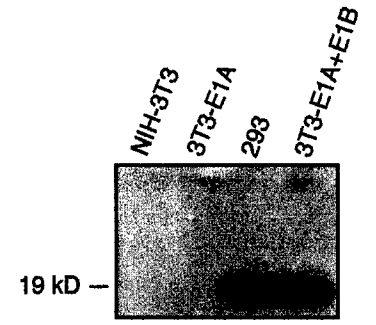


FIG. 5. NIH-3T3 cells selected for studies of the effects of E1A 19 kDa protein expression on E1A sensitization to injury-induced apoptosis express a high level of E1B 19 kDa oncoprotein. Quantitative immunoblot analysis of E1B 19 kDa protein expression in an NIH-3T3 cell clone (E1PB9) selected for coexpression of E1A and E1B contrasted with E1B 19 kDa expression in 293 cells which were known to stably express E1B 19 kDa at a high level. Nontransfected NIH-3T3 cells and NIH-3T3 cells transfected with only the E1A gene (3T3-E1A) served as E1B-negative controls.

E1A sensitization to CL-induced apoptosis in a single NIH-3T3 cell line.

Several possibilities were considered to explain this failure of apoptosis blockade by E1B, including inadequate E1B 19 kDa protein expression, another property unique to that cell line (E1PB9) which could prevent E1B 19 kDa from blocking apoptosis, and excessive CL-induced cell injury which prevented detection of limited E1B 19 kDa blockade. E1B 19 kDa protein expression level in E1PB9 cells was compared quantitatively to that detected in the E1A+E1B-expressing human cell line, 293 [39], which, in our comparison immunoblotting studies, consistently expresses E1B 19 kDa at the highest level among several adenovirus-transformed or oncogene-transfected mammalian cell lines. E1PB9 expressed a steady state level of E1B 19 kDa protein that was comparable to that of 293 cells (Fig. 5). Therefore, low-level expression of E1B 19 kDa protein in E1PB9 did not explain the failure of E1B to block CL-induced apoptosis of these E1A-sensitized cells.

Experiments were done to test the possibilities that the failure of E1B 19 kDa blockade was caused by another trait (unrelated to E1B protein expression level) unique to the E1PB9 or to NIH-3T3 cells. We also tested the possibility that the high killer cell to target cell ratio used in the studies shown in Table 1 created a proapoptotic injury that could not be overcome by E1B 19 kDa. For these purposes, we tested different target cell types and a range of CL to target cell ratios. Mouse (10)3 fibroblasts expressing either E1A alone or E1A+E1B 19 kDa were compared with similar NIH-3T3 clones (Fig. 6). Even at low CL to target cell ratios, there was no significant, E1B-related reduction in the apoptotic sensitivity of either type of E1A-positive mouse fibroblast (Figs. 6A and 6B). In fact there was a

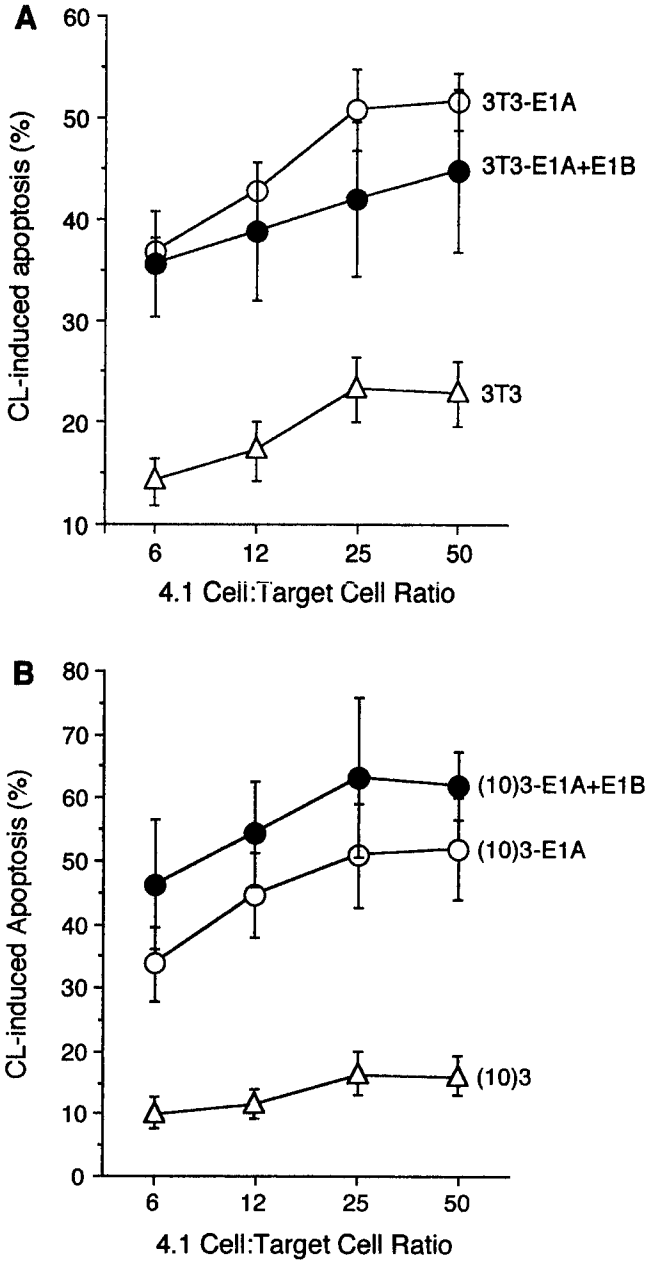


FIG. 6. E1B 19 kDa coexpression does not block CL-induced apoptosis of either E1A-expressing NIH-3T3 cells or E1A-expressing (10)3 cells across an eightfold range of killer cell to target cell ratios. NIH-3T3 (A) and (10)3 (B) cells expressing either E1A alone or E1A + E1B 19 kDa were compared with nontransfected parental cells for sensitivity to CL-induced apoptosis using the mouse CTL clone, 4.1, as the source of CL-induced injury. Points represent the mean ± SEM of three experiments with each set of target cells. The sensitivity to CL-induced apoptosis of NIH-3T3 or (10)3 cells coexpressing E1A + E1B 19 kDa was not significantly different from that of the cells of the same type expressing E1A alone. Both NIH-3T3 and (10)3 cells expressing either E1A alone or E1A + E1B 19 kDa proteins were significantly more sensitive to CL-induced apoptosis than the respective, E1A-negative parental cells ($P < 0.05$).

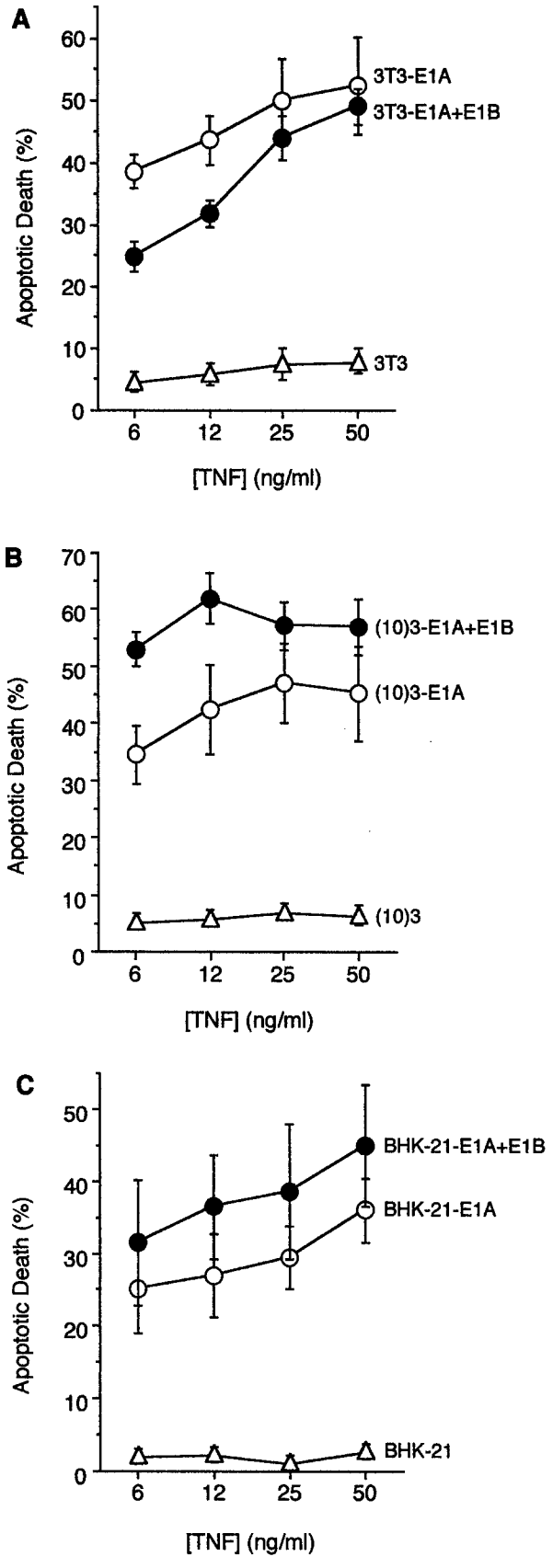
trend toward increased CL-induced apoptosis of the E1A+E1B-positive (10)3 cells vs the E1A-only-expressing (10)3 cells across the whole range of E:T ratios (Fig. 6B). These findings were consistent with our reports that E1A-sensitized, BHK-21 hamster cells are no less sensitive to CL-induced killing when they coexpress high levels of E1B 19 kDa [26] and that human 293 and HE1-E1 cells, both of which coexpress E1A and high levels of E1B 19 kDa, are highly sensitive to CL-induced killing [71].

The possibility that cell-type- or species-specific differences could explain the lack of E1B 19 kDa blockade of TNF-induced apoptosis in E1A-expressing cells was also tested. The same sets of NIH-3T3 and (10)3 cells tested against CL-induced injury were compared with the BHK-21 cell lines, BHK-21 clone 13.8 (no oncogene expression), BHK-B2 (E1A expression only), and BHK-D5 (E1A + E1B coexpression) [26] (Fig. 7). E1B 19 kDa-related differences in TNF-induced apoptosis in these experiments varied with both the cell type and the TNF concentration tested. For example, in comparison studies of NIH-3T3 cells, E1B 19 kDa coexpression resulted in a significant reduction in the apoptotic sensitivity of E1A-positive cells at low (6 and 12 ng/ml), but not high (25 and 50 ng/ml), TNF concentrations (Fig. 7A). This result suggested that a weak blockade of TNF-induced apoptosis can be detected *in vitro,* but that this E1B effect is overcome at higher TNF concentrations. Experiments with (10)3 mouse cells and BHK-21 hamster cells showed no blocking effect of E1B 19 kDa against TNF-induced apoptosis of E1A-sensitized cells. Indeed, with both of these types of cells, there was a trend toward increased TNF-induced apoptosis with E1A + E1B-expressing cells compared with cells expressing E1A alone (Figs. 7B and 7C).

DISCUSSION

These studies show that the mechanism by which the E1A oncoprotein sensitizes mammalian cells to immune-mediated apoptosis does not require expression of p53 in either rodent fibroblasts (Fig. 1) or human tumor cells (Fig. 2). These results suggest that E1A-induced sensitization to immune-mediated apoptosis should be effective in other rodent or human tumor cells that lack p53 or in which p53 mutations render the antioncogene nonfunctional. In contrast, these data (Table 1) and those of others [54, 55] indicate that triggering of apoptosis by certain chemical injuries can require p53 expression, even if the cells being tested are highly sensitive to p53-independent, proapoptotic injuries.

The CL assays used for these studies were done so that the apoptosis of E1A-positive target cells was caused by degranulation-dependent injury and not by injury caused by CL-expressed Fas ligand [24]. This



distinction separates the mechanism of CL-induced injury that was tested here from the triggering mechanism involved in TNF-induced apoptosis, which is analogous to the Fas mechanism [reviewed in 2]. TNF triggering of apoptosis is caused by binding of this cytokine to its cell surface, heterodimeric receptors that activate the caspase cascade through TNF-receptor-associated proteins [reviewed in 2].

The chemical injuries tested were also selected because of the differences in their mechanisms of triggering apoptosis. For example, hygromycin is a protein synthesis inhibitor that likely initiates apoptosis through a mechanism that is completely different from that of either CL or TNF. Although it is not known how these three different types of injuries converge to cause cellular apoptosis, it is clear that none of them requires p53 expression to induce apoptosis in E1A-sensitized mouse fibroblasts (Table 1 and Fig. 4A). In contrast, apoptosis induced in E1A-positive cells by three other, diverse types of chemical injuries—beauviricin (potassium ionophore), etoposide (topoisomerase II inhibitor) and H_2O_2 (source of reactive oxygen intermediates)—was strictly p53-dependent (Table 1). The diversity of the injury mechanisms that can trigger apoptosis in E1A-positive cells strongly suggests that the mechanism of E1A-induced sensitization does not involve any one specific type of apoptosis initiating signal. Instead,

FIG. 7. E1B 19 kDa coexpression had a variable effect on TNF-induced apoptosis of E1A-positive cells mouse and hamster cells that was cell-type-specific and was eliminated at high TNF concentrations. NIH-3T3 cells (A), (10)3 cells (B) (both mouse fibroblast cell lines), and BHK-21 hamster cells (C) expressing E1A alone or coexpressing E1A + E1B 19 kDa were tested for sensitivity to apoptosis induced by recombinant, mouse TNF at the indicated TNF concentrations. Points represent the mean ± SEM results of five (A), six (B), or four (C) experiments. There was a TNF-concentration-dependent effect of E1B 19 kDa expression on TNF-induced apoptosis of E1A-positive mouse cells. (A) At low TNF concentrations (6 and 12 ng/ml) E1B 19 kDa coexpression was associated with *reduced* sensitivity of E1A-positive, NIH-3T3 cells ($P < 0.05$), whereas at high TNF concentrations (25 and 50 ng/ml), there was no significant difference in the TNF sensitivity of cells expressing E1A + E1B 19 kDa vs E1A alone. (B) The results with (10)3 cells were opposite to those with NIH-3T3 cells at low TNF concentrations where E1B 19 kDa coexpression was associated with *increased* sensitivity of E1A-positive cells to TNF-induced apoptosis ($P < 0.05$). As had been observed with the NIH-3T3 lines, there was no significant difference in the sensitivity of (10)3 cells expressing E1A + E1B 19 kDa vs E1A alone at high concentrations of TNF. (C) In contrast to the results with the two mouse cell lines, there was no significant difference in the sensitivity of BHK-21 cells to TNF-induced apoptosis resulting from E1B 19 kDa coexpression with E1A vs cells expressing E1A alone. There was a consistent trend toward increased TNF sensitivity of the cells coexpressing E1A + E1B kDa, however, at all TNF concentrations tested, cells of all three types that either expressed E1A alone or E1A + E1B 19 kDa proteins were significantly more sensitive to TNF-induced apoptosis than the respective, nontransfected control cells ($P < 0.05$).

these results favor the conclusion that E1A affects a later stage in the cellular response to apoptotic injury.

The marked difference among proapoptotic agents in the p53 requirement to initiate injury of E1A-sensitized cells raises questions about the role of p53 in apoptosis initiation. Perhaps the best known example of the variable role of p53 in the injury-initiation phase of apoptosis is from mouse thymocyte studies [13, 56]. DNA damage of thymocytes caused by ionizing radiation is p53-dependent for inducing apoptosis, whereas glucocorticoids and calcium-dependent activating agents that mimic T cell receptor engagement induce apoptosis independently of p53 expression. Growth factor withdrawal from mouse lymphoma cells can also require p53 to initiate cellular injury [38]. These data support the conclusion that it is the cellular mechanism of injury initiation that is p53-dependent, not the inherent apoptotic sensitivity of the thymocytes.

We propose this same interpretation of the E1A data presented here. Our results suggest that it is the apoptosis initiation mechanism that either is or is not dependent on p53 expression, not the cellular mechanism through which E1A sensitizes cells to apoptotic injury. This could reconcile apparent differences between our results and the conclusions of previous reports on the role of p53 in E1A sensitization of mouse cells to injury-induced apoptosis. Studies by Lowe and colleagues indicated that E1A sensitization of mouse fibroblasts to apoptosis is dependent on p53 expression [54, 55]. Ionizing radiation, chemotherapeutic agents (including etoposide), and serum withdrawal were used as the proapoptotic stimuli in their experiments. In our experiments, etoposide-induced apoptosis of E1A-positive mouse cells required p53 expression, but the same E1A-positive, p53-negative cells that were resistant to etoposide-induced apoptosis were highly sensitive to apoptosis induced by CL or TNF (Table 1). It is possible that the E1A-positive, p53-negative mouse cells described by Lowe *et al.* [54, 55] also would have been found to be susceptible to apoptosis if CL and TNF had been tested as proapoptotic injuries. This study comparison underscores the importance of testing a broad range of proapoptotic stimuli, including CL and TNF α, when assessing the requirement for p53 in determining the apoptotic sensitivity or resistance of E1A-expressing cells.

Creation of stable, E1A expressing cell lines, such as those used in these studies, involves extensive cell selection. It has been argued that selection of E1A-positive cells during cloning could require collateral, cellular gene mutations [88]. Therefore, it is possible that one or more such mutations is required for the apoptosis-sensitive phenotype observed with the E1A-positive cells tested in our studies. However, other data from this laboratory indicate that cell-selection-related mutations are not required for E1A-induced sensitization to apoptotic injury. We have observed that transient expression of E1A oncoproteins, without cell selection, results in exactly the same E1A-induced sensitization to apoptotic injury as is reported here with the stable, E1A-expressing cells. In one such experiment, p53-positive NIH-3T3 cells were induced to express E1A following IPTG treatment. These cells exhibited the same E1A-induced sensitization to immune-mediated (J. L. Cook, unpublished data) and chemical [16] apoptotic injuries that was observed in the studies presented here. In a second type of experiment, NIH-3T3 cells transiently transfected with an E1A expression plasmid were highly sensitive to TNF-induced apoptosis within days after transfection and without any cell selection (J. L. Cook, unpublished data). Therefore, it is unlikely that any cellular mutations are required to complement E1A activities to induce sensitization to apoptotic injuries.

There have been other reports of p53-independent, E1A-induced apoptosis. However, most of these observations appear to be related to an indirect effect of E1A, resulting from its activation of an Ad E4 ORF4 gene product which, in turn, induces p53-independent apoptosis through a mechanism that remains to be defined [11, 49, 57, 81, 85]. In another report of TNF-induced, p53-independent apoptosis in which the target cells expressed E1A [47], the cells also expressed a mutant *ras* oncogene that has been reported to variably increase or decrease the cellular apoptotic response [5–7, 34, 35, 45, 61, 91]. Indeed, mutant ras expression itself can cause p53-independent apoptosis [58]. Therefore, it is difficult to assess the independent role of E1A in sensitizing cells to proapoptotic stimuli when the cells coexpress mutant *ras*.

The antiapoptotic activity of E1B 19 kDa was either completely or relatively ineffective in blocking the p53-independent, proapoptotic injuries inflicted by CL or TNF on E1A-sensitized cells in these studies (Figs. 4, 6, and 7). These results are consistent with our reported observations that E1B gene expression does not block E1A sensitization to CL-induced killing of Ad-infected hamster cells, Ad-transformed hamster, rat, or human cells, or stably E1A-transfected BHK-21 cells [19, 20, 25, 26, 50, 71, 90]. Others have also reported that E1B coexpression does not block TNF-induced apoptosis of E1A-expressing rat or mouse cells [48, 87].

These observations can be considered in the context of information on the mechanisms by which CL and TNF trigger apoptosis and E1B 19 kDa blocks this response to provide insight into possible mechanisms by which E1A sensitizes cells to immune-mediated apoptosis. Degranulation-dependent killing by CL triggers the apoptosis cascade primarily through the actions of granzyme B, which directly activates several cellular caspases, including caspases 8 and 3 [28, 36, 59, 64, 70]. TNF triggering of apoptosis is initiated by

its binding and trimerization of the TNF receptor which, in turn, activates the caspase cascade at the level of caspase 8, through an intermediary protein, FADD [reviewed in 2]. Relatively little is known about the mechanisms by which E1B 19 kDa blocks apoptosis. However, it is reported that E1B 19 kDa interferes with the function of FADD in its activating interactions with caspase 8 [65]. There are also data suggesting that E1A might activate caspase 8 [63] and that the kinetics of cell injury-induced activation of caspase 8 can be cell-type-specific [76]. It is apparent from these observations that several molecular interactions in the initiation of the caspase cascade could influence the balance between injury-induced activation of apoptosis, E1A enhancement of this activation, and the ability of E1B to block it. There is also evidence that CL injury can completely bypass the caspase activation sequence to induce apoptosis [74, 86]. Therefore, it is possible that E1A activates a step in this less well defined, "postcaspase" stage in the cellular apoptotic response. Another possibility is that E1A might sensitize cells to immune-mediated, proapoptotic injuries by reducing the cellular, antiapoptotic defense, rather than by enhancing one or more steps in the caspase cascade or postcaspase apoptosis activation response. Considering the multiple molecular activities that have been linked with E1A expression, it is likely that the final answer regarding the mechanism(s) of E1A-induced cellular sensitization to apoptosis will involve alterations at multiple steps in the cellular response to proapoptotic injury.

These results and previously reported data can be used to consider the implications of E1A-induced sensitization to immune-mediated apoptosis and E1B 19 kDa antiapoptotic activity for tumor formation in immunocompetent animals. The results in Figs. 1, 2, 4, and 6 and previously published data [25, 26, 71, 90] indicate that E1A expression sensitizes both rodent and human tumor cells to CL-induced apoptosis and that E1B 19 kDa expression cannot block this E1A activity. The data in Fig. 7 show that the same E1A-induced sensitization and lack of E1B 19 kDa blockade occur with TNF-treated hamster sarcoma cells. We reported that Ad-transformed cells that express high levels of both E1A and E1B 19 kDa proteins are highly susceptible to killing by activated macrophages [18, 19, 21, 22, 25, 50] which use TNF as a major cytotoxic factor against E1A-expressing cells [23]. We have also observed that hamster BHK-21 sarcoma cells that co-express E1A + E1B 19 kDa proteins are rejected by immunocompetent animals as efficiently as BHK-21 cells that express only E1A [26]. These *in vivo* data are consistent with many reports that Ad2- and Ad5-transformed rodent cells, most of which are likely to express high levels of both E1A and E1B 19 kDa proteins, are unable to form tumors in immunocompetent animals [reviewed in 51] and that E1A expression causes apoptosis in tumor cells *in vivo* [30]. These data suggest that E1B 19 kDa antiapoptotic activity that can be detected in some *in vitro* assays is not a significant factor *in vivo* in determining the tumorigenicity of E1A-expressing tumor cells in immunologically intact animals.

Collectively, the observations in this report indicate that the mechanisms by which E1A sensitizes tumor cells to immune-mediated apoptosis and to rejection by the cellular immune response *in vivo* does not require cellular expression of wild-type p53 and can function independently of the Bcl-2-like, antiapoptotic activities of E1B 19 kDa. The precise molecular mechanism(s) of E1A-induced, p53-independent sensitization of cells to apoptosis triggered by immune-mediated injuries and other proapoptotic stimuli remains to be defined.

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Adenovirus E1A Oncogene Expression in Tumor Cells Enhances Killing by TNF-Related Apoptosis-Inducing Ligand (TRAIL)[1]

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Expression of the adenovirus serotype 5 (Ad5) E1A oncogene sensitizes cells to apoptosis by TNF-α and Fas-ligand. Because TNF-related apoptosis-inducing ligand (TRAIL) kills cells in a similar manner as TNF-α and Fas ligand, we asked whether E1A expression might sensitize cells to lysis by TRAIL. To test this hypothesis, we examined TRAIL-induced killing of human melanoma (A2058) or fibrosarcoma (H4) cells that expressed E1A following either infection with Ad5 or stable transfection with Ad5-E1A. E1A-transfected A2058 (A2058-E1A) or H4 (H4-E1A) cells were highly sensitive to TRAIL-induced killing, but Ad5-infected cells expressing equally high levels of E1A protein remained resistant to TRAIL. Infection of A2058-E1A cells with Ad5 reduced their sensitivity to TRAIL-dependent killing. Therefore, viral gene products expressed following infection with Ad5 inhibited the sensitivity to TRAIL-induced killing conferred by transfection with E1A. E1B and E3 gene products have been shown to inhibit TNF-α- and Fas-dependent killing. The effect of these gene products on TRAIL-dependent killing was examined by using Ad5-mutants that did not express either the E3 (H5*dl327*) or E1B-19K (H5*dl250*) coding regions. A2058 cells infected with H5*dl327* were susceptible to TRAIL-dependent killing. Furthermore, TRAIL-dependent killing of A2058-E1A cells was not inhibited by infection with H5*dl327*. Infection with H5*dl250* sensitized A2058 cells to TRAIL-induced killing, but considerably less than H5*dl327*-infection. In summary, expression of Ad5-E1A gene products sensitizes cells to TRAIL-dependent killing, whereas E3 gene products, and to a lesser extent E1B-19K, inhibit this effect. *The Journal of Immunology,* **2000, 165: 4522–4527.**

Human adenoviruses (Ad)[3] are common human pathogens. In immunocompetent people, Ad cause persistent, but self-limited infections (1, 2). Although not oncogenic in humans, Ad are capable of transforming human cells in vitro to a state where the cells are tumorigenic in immunocompromised rodents (3, 4). Cells transformed by Ad are virion free and only two viral genes (E1A and E1B) are consistently expressed (5). The primary immortalizing gene is E1A. E1B serves as a "helper" gene that increases the efficiency of viral transformation (5).

One possible explanation for the lack of oncogenicity of Ad in humans is the capacity of E1A to sensitize cells to destruction by components of the host cellular immune response (6–9). The expression of E1A sensitizes cells to lysis by components of the cellular antitumor immune response, including NK cells, activated macrophages, and CTL (6–9). These effector cells kill target cells by several mechanisms including the elaboration of secreted proteins, such as TNF-α and perforin, or by the interaction of Fas ligand on effector cells with Fas on target cells. The expression of E1A in epithelial or fibroblastic cells induces an increased susceptibility to lysis by TNF-α, perforin, and Fas ligand (10–12).

Recent studies have characterized a new member of the TNF family of cytokines, the TNF-related apoptosis-inducing ligand (TRAIL) (13, 14). TRAIL interacts with receptors that are distinct from the receptors for Fas ligand and TNF-α. To date, five receptors have been shown to bind TRAIL. The binding of TRAIL to either TRAIL-R1 (death receptor 1) or TRAIL-R2 induces apoptosis (15–17). In contrast, the binding of TRAIL to TRIAL-R3, TRAIL-R4, or osteoprotegerin does not induce apoptosis (15, 16, 18–21). TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and osteoprotegerin are all members of the TNF-receptor family. The cytoplasmic domains of TRAIL-R1 and TRAIL-R2, like those of the receptors for Fas ligand and TNF-α, contain cytoplasmic death domains and likely use the same or similar pathways of caspase activation to induce apoptosis (22). In contrast, TRAIL-R3 and TRAIL-R4 lack cytoplasmic death domains, and osteoprotegerin is a secreted protein. In contrast to TNF-α and Fas ligand, which are expressed primarily by cells of the immune system, TRAIL is produced by a wide variety of cell types (22). TRAIL preferentially induces apoptosis of certain tumor cell lines and virally infected cells, whereas nontransformed cells are generally resistant to TRAIL-induced killing (23, 24). TRAIL also contributes to the cytotoxicity mediated by $CD4^+$ T cells and monocytes (25).

Based on the ability of TRAIL to selectively kill virally infected cells and the similar molecular mechanisms of TRAIL-, Fas-, and TNF-α-induced apoptosis, we postulated that E1A expression in Ad-infected and E1A-transfected tumor cells might also sensitize

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[3] Abbreviations used in this paper: Ad, adenovirus; TRAIL, TNF-related apoptosis-inducing ligand.

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cells to TRAIL-induced killing. To test this hypothesis, we determined the effect of E1A expression on TRAIL-induced killing of A2058 and H4 cells. A2058 cells, a human melanoma cancer cell line, and H4, a human fibrosarcoma cell line, were chosen because they are resistant to TRAIL-induced killing and are permissive for infection with Ad5 (26). We found that the expression of E1A gene products sensitizes human tumor cells to TRAIL-dependent killing. The ability of E1A to sensitize cells to TRAIL-induced killing following Ad5 infection was inhibited by E3 gene products, and to a lesser extent E1B-19K.

Materials and Methods

Cells and cell lines

A2058 is a human melanoma cell line. H4 is a human fibrosarcoma cell line. A2058-E1A (also known as 1A558C8) and H4-E1A (also known as P2AHT2A) are Ad5-E1A-transfected A2058 and H4 cells, respectively (27). Cell lines were maintained in DMEM supplemented with 5% calf serum, glucose (15 mM), antibiotics, and glutamine. The absence of *Mycoplasma* in the cell lines was established by using the Mycotect Assay (Life Technologies, Gaithersburg, MD).

Viruses

H5*dl327* is an Ad5 mutant virus that does not express any E3 proteins (28). H5*dl250* is an Ad5 mutant that does not express the E1B-19K protein (29). Wild-type Ad5 and H5*dl327* were grown in A549 cells. H5*dl250* was grown in 293 cells. Each virus was titered by plaque assay using the cell line in which it was grown.

TRAIL cytolysis assays

Target cells were infected with the different Ad (multiplicity of infection = 100) for 16 h and labeled with ^{51}Cr (100 mCi/ml for 1 h; 1 Ci = 37 gBq). Initial studies showed that equivalent amounts of E1A were expressed following infection of cells at a multiplicity of infection of 100 with wild-type or mutant forms of Ad5 (data not shown). Target cells (1×10^4 cells) were then incubated with different concentrations of recombinant human TRAIL (R&D Systems, Minneapolis, MN). After a 16-h incubation at 37°C in 5% CO_2, half of the supernatant from each well was harvested and counted in a gamma counter. TRAIL-dependent killing was determined by calculating the percentage of TRAIL-induced release of radiolabel from target cells as described (9). The results shown represent the mean ± SEM of at least four separate experiments. The mean percentage spontaneous release from all types of target cells was <30%. The significance of the differences in TRAIL-induced killing of control and infected cell lines was estimated using Student's *t* test.

Western analysis

For quantitation of E1A proteins, 60-mm plates of Ad-infected or E1A-transfected A2058 or H4 cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl), and protein concentrations of RIPA supernatants were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL). An equal amount of protein from each cell lysate was separated on 10% SDS polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% nonfat milk solution and incubated with the anti-E1A mAb, M73 (supplied by E. Harlow, Massachusetts General Hospital, Charleston, MA) for 1 h (30). Following several washes with PBST (PBS with 0.05% Triton X-100), membranes were incubated for 1 h with rabbit anti-mouse IgG Ab (Cappel, Durham, NC) and washed extensively with PBST. The E1A protein was then visualized as per manufacturer instructions using the Renaissance Chemiluminescence Kit (DuPont-NEN, Boston, MA).

Results

E1A-transfection, but not infection, with wild-type Ad5 sensitizes tumor cells to TRAIL-induced killing

To determine whether E1A sensitized cells to TRAIL, cytolysis assays were performed on parental and E1A-transfected melanoma (A2058, A2058-E1A) and fibrosarcoma (H4, H4-E1A) cells. In agreement with prior reports, we found that both H4 and A2058 cells were resistant to TRAIL-induced killing (Fig. 1). The resistance of A2058 or H4 cells to TRAIL-induced killing was abrogated by the stable transfection with E1A (Fig. 1). Cell lines have been considered sensitive to TRAIL-induced killing if incubation of 300 ng/ml of recombinant TRAIL results in >20% killing (22, 26). We found that the addition of 12.5–25 ng/ml of TRAIL killed >25% of A2058-E1A and H4-E1A cells. The ability of E1A expression to sensitize tumor cells to TRAIL-dependent killing was not restricted to melanoma or fibrosarcoma cells. TRAIL-dependent killing of breast cancer and cervical cancer cell lines was also increased by stable E1A-transfection (data not shown). Next, we ascertained whether the expression of E1A gene products following Ad5 infection would also induce TRAIL-dependent killing. In contrast to transfection with E1A, infection of A2058 or H4 cells with Ad5 did not confer sensitivity to TRAIL-dependent killing (Fig. 1).

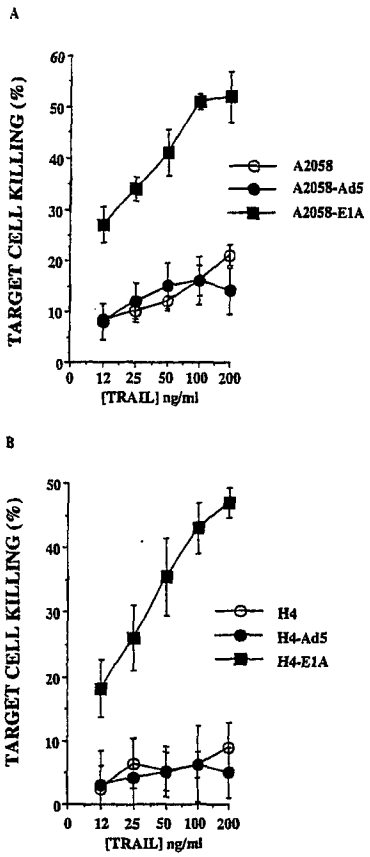

FIGURE 1. E1A-transfected, but not Ad5-infected, human tumor cells are sensitive to TRAIL-induced lysis. A2058 and A2058-E1A are parental and E1A-transfected, human melanoma cells, respectively. H4 and H4-E1A are parental and E1A-transfected fibrosarcoma cells. *A*, TRAIL-induced lysis of A2058 (○), Ad5-infected-A2058 (●), or A2058-E1A (■) cells. *B*, TRAIL-induced killing of H4 (○), Ad5-infected H4 (●), or H4-E1A (■) cells.

E1A oncoprotein expression is equivalent in E1A-tranfected or Ad5-infected A2058 cells

The ability of E1A gene products to increase the susceptibility to lysis by NK cells and macrophages is dependent on the level of E1A expressed (11). Prior studies from our laboratory showed that equivalent amounts of E1A protein are expressed in Ad5- compared with E1A-transfected H4 cells (6). However, it was possible that the levels of E1A produced following Ad5-infection of A2058 cells were insufficient to induce sensitivity to TRAIL-dependent killing. To test this possibility, the levels of E1A oncoprotein in Ad5-infected and E1A-transfected A2058 cells were compared. Western blot analysis of cell lysates demonstrated that the amounts

of E1A in Ad5-infected and E1A-transfected A2058 cells were comparable (Fig. 2). Therefore, the resistance of Ad5-infected A2058 or H4 cells to TRAIL-dependent killing could not be explained by quantitative differences in the expression of E1A gene products.

Inhibition of TRAIL-dependent killing by E1B and E3 gene products

Expression of E1A gene products in immortalized cells induces susceptibility to NK cell lysis. In contrast, infection of human cells with Ad does not render the cells susceptible to NK cell lysis (6). Thus, it was possible that the transient expression of E1A gene products following Ad5-infection was insufficient to induce sensitivity to TRAIL-dependent killing. Alternatively, other viral gene products produced following Ad infection could be responsible for the inability of Ad5 infection to sensitize cells to TRAIL-dependent killing. To test the latter possibility, we examined whether Ad5-infection inhibited TRAIL-dependent killing of A2058-E1A cells. As shown in Fig. 3, Ad5-infection of A2058-E1A cells reduced the killing by TRAIL compared with that obtained with uninfected cells.

Adenoviral E1B and E3 gene products inhibit apoptosis induced by Fas ligand and TNF-α The effect of these gene products on TRAIL-dependent killing was examined by using mutant strains of Ad5 that did not express either the E3 (H5*dl327*) or E1B-19K (H5*dl250*) coding regions. A2058 cells infected with H5*dl250* (which does not express E1B-19K, but does express E1A and E3 proteins) resulted in a small increase in TRAIL-induced killing compared with uninfected cells (Fig. 4*A*). In contrast, infection of A2058 cells with H5*dl327* (which does not express E3 proteins, but does express E1A and E1B-proteins) increased the sensitivity of A2058 cells to TRAIL-dependent killing to a level that was similar to that observed with A2058-E1A cells (Fig. 4*A*). Furthermore, infection of A2058-E1A cells with H5*dl327* did not inhibit their TRAIL sensitivity (Fig. 4*B*). The ability of *H5dl250* infection to block TRAIL-induced killing in A2058-E1A cells could not be assessed because infection of these cells with H5*dl250* produced a high spontaneous release of ^{51}Cr. In total, these data indicated that E3 proteins inhibited the ability of E1A gene products, produced following transfection or infection, to sensitize cells to TRAIL-dependent killing. The E1B-19K protein inhibited TRAIL-induced killing to a lesser extent than E3 gene products.

Discussion

In this study, we demonstrate that expression of the Ad5-E1A oncoprotein following transfection of the E1A gene sensitized melanoma (A2058) and fibrosarcoma (H4) tumor cells to lysis by TRAIL (Fig. 1). Similar data were obtained with E1A-transfected breast cancer and cervical cancer cells (data not shown). These data are similar to reports that E1A gene products sensitize human and murine cells to TNF-α and Fas-dependent killing (10–12). However, E1A gene products do not always sensitize human or rodent cells to lysis by immune mediators or effector cells (8, 31–33). Furthermore, the molecular basis for the increased susceptibility of E1A-expressing cells to TNF-α and Fas-dependent killing is unknown. Therefore, the ability of E1A to sensitize human tumor cells to TRAIL-dependent killing needed to be directly ascertained.

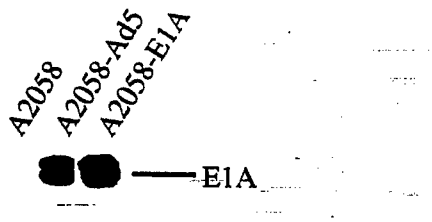

FIGURE 2. Expression of E1A in Ad5-infected compared with E1A-transfected human tumor cells. A2058 cells were infected with Ad5 for 16 h and the levels of E1A oncoprotein expression were determined by Western analysis (see *Materials and Methods*). A2058-E1A is an E1A-transfected A2058 cell line.

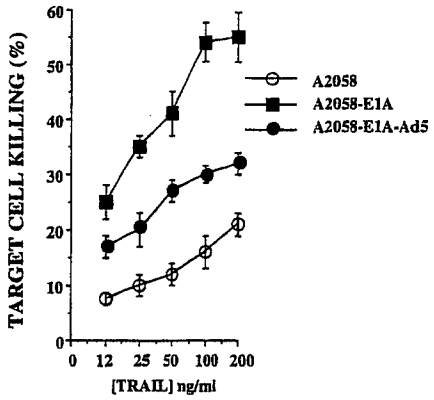

FIGURE 3. Ad5-infection inhibits TRAIL-dependent killing of E1A-transfected cells. Parental and E1A-transfected A2058 cells were mock or Ad5-infected for 16 h and their sensitivity to TRAIL-induced killing was determined in a 16-h cytolysis assay. TRAIL-induced lysis of A2058 (○), Ad5-infected A2058-E1A (●), or uninfected A2058-E1A cells (■).

TRAIL-dependent killing of A2058 cells was also increased by the expression of E1A gene products produced in the context of Ad-infection. However, because E1B and E3 gene products block TRAIL-induced killing, this effect of E1A could not be detected by infection of A2058 cells with wild-type Ad5. For example, in contrast to A2058-E1A cells, A2058 cells infected with wild-type Ad5 were resistant to lysis by TRAIL (Fig. 1*A*). This difference between Ad5-infected and E1A-transfected cells was not due to differences in E1A expression. A2058-E1A cells and A2058 cells infected with Ad5 expressed equivalent levels of E1A oncoprotein (Fig. 2). Similar to results on A2058 cells, Ad5-infected H4 cells also were resistant to TRAIL-dependent killing, despite high levels of E1A expression (Fig. 1*B*, and Ref. 6). Proteins encoded by the E3 regions were predominately responsible for the resistance of Ad5-infected A2058 cells to lysis by TRAIL. A2058 cells infected with H5*dl327* (which does not express E3 proteins, but does expresses E1B proteins) were nearly as sensitive to TRAIL-dependent killing as A2058-E1A cells (Fig. 4*A*). TRAIL-dependent killing was also blocked by E1B-19K, although they were less effective than proteins encoded by the E3 region. Thus, H5*dl250* (which does not express E1B-19K, but expresses E3 proteins) infection of A2058 cells increased TRAIL-dependent killing, but to a lesser extent than infection with H5*dl327* (Fig. 4*A*). Furthermore, E1B gene products failed to inhibit killing of A2058-E1A cells following infection with H5*dl327* (Fig. 4*B*). In contrast, TRAIL-dependent killing of A2058-E1A cells was blocked following infection with Ad5 (expresses E1B and E3; Fig. 4). In total, these studies indicate that E3 gene products were more effective at inhibiting TRAIL-dependent killing than the E1B-19K proteins.

Ad encode numerous proteins that facilitate the evasion of the host immune response and contribute to viral persistence (34). Several proteins encoded by the E1B and E3 regions have been previously shown to inhibit TNF-α and Fas-induced killing and

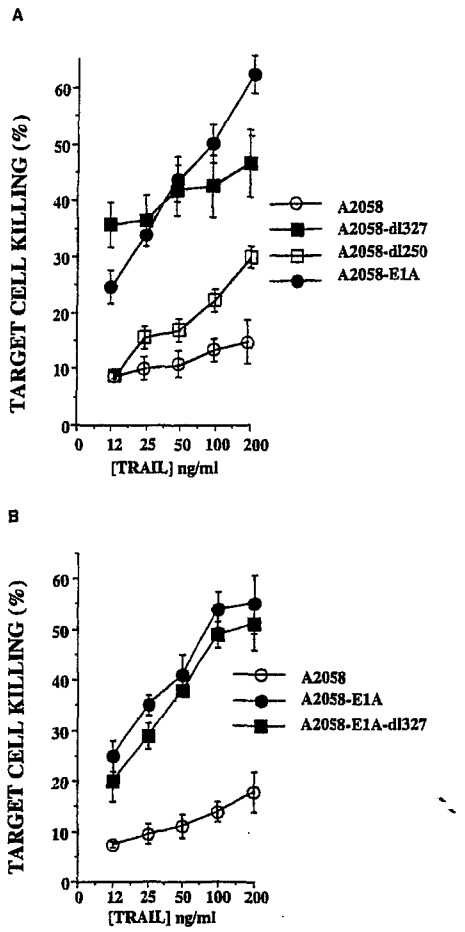

FIGURE 4. E1B and E3 gene products inhibit TRAIL-dependent killing. H5*dl250* and H5*dl327* are Ad5-mutants that delete the E1B-19K and E3 open reading frames, respectively. Tumor cell lines were mock-, H5*dl250*-, or H5-*dl327*-infected for 16 h and their sensitivity to TRAIL-induced killing was determined in 16-h cytolysis assays. *A*, TRAIL-induced killing of A2058 (○), A2058-E1A (●), H5*dl327*- (■), or H5*d/250*-infected (□) A2058 cells. *B*, TRAIL-induced killing of A2058 (○), H5*dl327*-infected (■), or unifected-A2058-E1A cells (●).

thus are candidates to inhibit TRAIL-induced killing (35–37). For example, the E1B 19K protein, which is functionally homologous to the protoncogene Bcl-2, inhibits the activation of Procaspace-8 (FLICE) (36). In addition, the E3 10.4K and 14.5K proteins (also known as E3-RID) bind to and reduce the surface expression of Fas (39, 40). Another E3 protein, E3–14.7K, blocks apoptosis by its ability to bind and alter the function of proteins involved in NF-κB-apoptosis regulatory pathway (41). Studies are ongoing to determine the molecular mechanism by which the E1B and E3 proteins inhibit TRAIL-induced killing.

Other adenoviral proteins are implicated in the evasion of host immune responses. Certain Ad-encoded proteins inhibit the biological activities of IFNs and CTL recogntion of Ad-infected cells (42–44). Therefore, it is puzzling that Ad rarely cause disseminated infections and do not appear to be oncogenic in humans, despite their well-described ability to transform human cells (4, 45–48). We speculate that the biological behavior of Ad in humans is best explained by the incomplete inhibition of the host cellular innate (NK cells, macrophages) and acquired (T cell) immune responses by adenoviral immunomodulatory proteins during infection as well as minimal effects of these viral proteins on immune defenses following viral transformation. For example, Ad-transformed human cells are selectively killed by resting and IFN-activated NK cells (6, 49). In contrast, Ad-infected human cells are only selectively killed by IFN-activated NK cells (33, 50). In mouse models, a robust CTL response is induced following injection of E1A-expressing tumor cells, and, as a result, CTL are highly effective at eliminating Ad-transformed cells in vivo (51, 52). We speculate that an effective CTL response would also be generated against E1A-expressing, Ad-transformed cells in humans. CTL are also induced following Ad-infection (53–55). However, CTL recognition of Ad-infected cells appears to be partially subverted by the Ad-E3-19K protein, a protein that is usually not expressed in Ad-transformed cells (44, 56, 57). The inhibition of TRAIL-dependent killing by the E3 proteins provides another mechanism that could enable Ad to escape innate immunity and favor viral persistence. However, our data suggest that TRAIL-dependent killing would be highly effective in eliminating E1A-expressing human cells that became immortalized following a transforming viral infection. For example, E1A-transfected A2058 and H4 cells and A2058 cells infected with H5*dl327* (which express the E1A and E1B proteins) were highly susceptible to TRAIL-dependent killing (Fig. 4). Therefore, we predict that Ad-transformed, E1A and E1B expressing human cells would also be highly sensitive to TRAIL-dependent killing. Thus, the immunomodulatory proteins encoded by Ad may prevent a rapid sterilizing immune response and favor viral-persistence, but are insufficient to fully evade host immunity. Furthermore, in Ad-transformed cells, these immunomodulatory proteins are either not present (E3) or are ineffective (E1B) in blocking killing by host effector killer mechanisms (5, 58).

The results from this study are also highly relevant in the selection of the optimal adenovirus mutant to be used in the treatment of human malignancy. Phase I clinical trials are presently underway using ONYX-015, an Ad-mutant that does not express the E1B-55K protein (59). E1B-55K partially inhibits the p53-dependent apoptosis induced by E1A-expression (60). The deletion of E1B-55K may enable ONYX-015 to replicate and induce apoptosis in p53-deficient human cancer cells but may leave normal (p53 positive) cells intact (61–63). For several reasons, we speculate that adenoviral mutants that contain deletions in both the E1B and E3 coding regions would be superior to ONYX-015 for the treatment of human malignancies. The first reason is that immune mechanisms likely participate in mediating tumor regression following Ad-infection. For example, several reports indicate that the ability of E1A to reduce tumorigenicity is dependent on NK and T cells (64–67). The ability of E1A to sensitize cells to immune-mediated apoptosis is independent of p53 (66, 68), insensitive to E1B-19K. The second reason is that prior studies indicate that combining chemotherapeutic agents with ONYX-015 is more effective in mediating tumor destruction than either agent alone (63). This effect is likely due to the ability of E1A to sensitize cells to apoptosis induced by chemotherapeutic agents (69). However, in E1A-expessing tumor cells, the p53-dependent induction of apoptosis induced by chemotherapeutic agents is also inhibited by E1B-19K (68). A third reason is that recent studies indicate that TRAIL may be useful in the treatment of human malignancies. By inducing the expression of TRAIL-R1 and TRAIL-R2, chemotherapeutic agents such as etoposide synergize with TRAIL to mediate tumor cell apoptosis (70). Studies reported here indicate that TRAIL-resistant tumors can be sensitized by the expression of E1A gene products. In contrast, E3 gene products block TRAIL-induced killing of E1A-expressing tumor cells. The use of mutant Ad that have deleted both E1B (19K and 55K) and E3 gene products may optimize the synergistic interactions involving chemotherapeutic agents, TRAIL and E1A. For all these reasons, we believe that mutant adenoviruses that contain deletions of both the

E1B and E3 open reading frames would be more effective in the treatment of human malignancies than ONYX-015, regardless of whether the virus was used alone or in combination with other chemotherapeutic agents. Studies are ongoing using murine models to directly test this hypothesis.

In summary, we show that the expression of E1A gene products sensitizes tumor cells to TRAIL-dependent killing. The gene products of the E3 region, and to a lesser extent E1B1-19K, inhibit this effect. Studies are ongoing to determine the molecular basis for the ability of E1A gene products to sensitize cells to TRAIL-dependent killing.

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Dissimilar Immunogenicities of Human Papillomavirus E7 and Adenovirus E1A Proteins Influence Primary Tumor Development

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Although human papillomaviruses (HPV) and adenoviruses (Ad) both transform cells by expressing functionally related oncogenes (Ad-E1A/E1B; HPV-E7/E6), only HPV are oncogenic in humans. Prior studies have shown that HPV-transformed cells are resistant to NK cell lysis and E7- and E6-specific CTL are inefficiently generated in women with HPV-induced cervical cancer. Therefore, we postulated that the dissimilar oncogenicities of Ad and HPV may be caused by a protective NK and T cell response that is triggered by transformed cells expressing E1A, but not by E7. To test this hypothesis, mice that were either immunologically intact, lacked T cells, or lacked both NK and T cells were challenged with Ad serotype 5 (Ad5)-E1A- or HPV16-E7-transfected tumor cells. E7-expressing tumor cells were resistant to NK cell lysis *in vitro* and failed to elicit a measurable anti-tumor NK or T cell response *in vivo*. The concomitant expression of E6 did not change this phenotype. In contrast, E1A-expressing tumor cells were sensitive to NK lysis *in vitro* and triggered a protective NK and T cell immune response *in vivo*. These data suggest differences in the capacities of E1A or E7 oncoproteins to trigger protective anti-tumor immune responses may contribute to the dissimilar oncogenicities of Ad and HPV in humans. © 2000 Academic Press

Key Words: adenoviruses; papillomaviruses; NK cells; E1A; E7.

INTRODUCTION

Human papillomaviruses (HPV)[2] and adenoviruses (Ad) are common human pathogens with a proclivity for causing persistent infections. In the United States, 95% of adults are seropositive for one of the group C Ad, and over 12 million adults are infected with genital tract HPV (Koutsky *et al.*, 1988; Straus, 1984). Although both viruses are competent to transform human cells, only HPV are oncogenic in humans (Bosch *et al.*, 1995; Green *et al.*, 1979).

In HPV-induced human malignancies or Ad-transformed cells, there is viral gene integration into the host genome, and expression of two viral genes (HPV, E6 and E7; Ad, E1A and E1B) is found consistently (Graham *et al.*, 1977; Schwarz *et al.*, 1985; Smotkin and Wettstein, 1986). E1A and E7 are the primary immortalizing genes of Ad and HPV, respectively. Ad-E1B and HPV-E6 serve as "helper" genes that increase the efficiencies of E1A- and E7-induced transformation (Halbert *et al.*, 1991; Houweling *et al.*, 1980). The E1A and E7 oncoproteins appear to transform cells by similar molecular mechanisms. Through two highly homologous, conserved regions [conserved region (CR) 1 and CR2], the E1A and E7 oncoproteins bind to and inhibit the function of common cell-growth regulatory proteins (e.g., p105 or retinoblastoma gene product, pRb; p107, p130, and cyclin A) (Dyson *et al.*, 1992; Phelps *et al.*, 1988; Vousden and Jat, 1989). Although genetically unrelated, Ad-E1B p55 and E6 oncoproteins derived from oncogenic HPV both inhibit the biological activities of the tumor suppressor gene p53 through different molecular mechanisms (Scheffner *et al.*, 1990; Yew and Berk, 1992; Zantema *et al.*, 1985). As a result of this activity, both E1B and E6 inhibit either E1A- or E7-induced cellular apoptosis and favor the survival of immortalized cells (Pan and Griep, 1994; Rao *et al.*, 1992).

HPV are frequently divided into two major classes based on their associations with cervical cancer. The "low-risk" types (HPV-6, HPV-11) cause benign cervical lesions. In contrast, "high-risk" HPV (HPV-16, -18, -31 and -33) are identified in over 95% of cervical carcinomas. The dissimilar oncogenicities of high- and low-risk HPV likely relate to the inability of low-risk HPV to transform human cells (Heck *et al.*, 1992; Münger *et al.*, 1991; Scheffner *et al.*, 1990). Unlike low-risk HPV, Ad are fully competent to transform human cells. This fact and the results of previous studies comparing other DNA tumor

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[2] Abbreviations used: Adenovirus, Ad; adenovirus serotype 5, Ad5; human papillomavirus, HPV.

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viruses indicate that there are factors other than cellular transformation efficiency that distinguish tumorigenic from nontumorigenic viruses. For example, although nononcogenic group C Ad (Ad serotype 2, Ad2, Ad5) and highly oncogenic group A Ad (Ad 12) transform rodent cells *in vitro* with similar efficiencies, only cells transformed by highly oncogenic serotypes form tumors in immunocompetent animals. Conversely, cells transformed *in vitro* by both types of viruses are tumorigenic in immunodeficient animals (Lewis and Cook, 1984). These and other data suggest that the outcomes of interactions between oncogene-expressing, virally transformed cells and the host cellular immune response are pivotal in determining whether primary tumors will or will not form *in vivo*.

In previous studies we showed that the oncogenicities of HPV- or Ad-transformed human cells correlated with their resistance to lysis by human NK cells (Routes and Ryan, 1995). Oncogenic, HPV transformed human cells were resistant to NK lysis, whereas Ad-transformed human cells were NK sensitive. The Ad-E1A and HPV-E7 oncoproteins regulated this difference in susceptibility to NK cell lysis. Additionally, several laboratories have reported that HPV-tumor-specific CTL responses are weak in women with cervical cancer (Borysiewicz *et al.*, 1996; Evans *et al.*, 1996; Ressing *et al.*, 1996). In contrast, studies in rodents show that robust CTL responses are induced following injection of Ad5-transformed cells (Bellgrau *et al.*, 1988). These data suggested to us that differences in the capacities of E7 and E1A proteins to target virally transformed cells for destruction by the cellular immune response might contribute to the dissimilar oncogenicities of HPV and Ad in humans. However, there previously had been no way to directly compare the immunogenicities of E1A- and E7-expressing tumor cells to determine whether oncoprotein immunogenicity could influence primary tumor development *in vivo*. Therefore, it was uncertain whether these *in vitro* findings were relevant to primary tumor development *in vivo*. (For these considerations, tumor cell immunogenicity is defined as the ability of the tumor cell to elicit an effective antitumor response *in vivo* that includes both innate (NK cell) and acquired (T cell) immune defenses).

To test the hypothesis that dissimilar NK and T cell responses directed against cells expressing E1A or E7 affect primary tumor formation, a murine model was developed using the C57/BL6-derived tumor cell line MCA-102. MCA-102 cells were chosen for several reasons. First, MCA-102 cells are highly oncogenic in immunocompetent animals. Second, MCA-102 cells are nonimmunogenic, even when transduced by the costimulatory molecules B7.1 or B7.2 (Chen *et al.*, 1994; Mule *et al.*, 1987; Yang *et al.*, 1995). Therefore, by transfecting the E1A and E7 oncogenes into MCA-102 cells, the immunogenicities of the E1A and E7 proteins could be directly compared and the relevance of oncoprotein immunogenicity on primary tumor formation could be assessed. Thus, if E1A were more immunogenic than E7, then immunocompetent mice should more efficiently reject MCA-102-E1A cells than MCA-102-E7 cells. Furthermore, if NK and T cells were important in the preferential rejection of E1A-expressing cells, the NK cell-related and T-cell-related components to the tumor rejection response could be quantitated through the use of mice that lack T cells (nude mice) or T cells and NK cells (CD3-ϵ-transgenic mice). Finally, because nontransfected MCA-102 cells were inherently resistant to killing by NK cells, it was also possible to test the effect of E1A and E7 oncogene expression on NK cell susceptibility *in vitro* and to correlate these results with *in vivo* tumor development data.

These results showed that the E1A- or E7-expressing mouse tumor cells exhibited phenotypes similar to those observed for virally transformed human tumor cells. E7-expressing cells were resistant to rejection by animals with competent NK cell and T cell responses and were resistant to NK cell lysis *in vitro*. Conversely, E1A-expressing cells were highly NK susceptible and over 1000 times less tumorigenic in immunocompetent mice than either E7-expressing cells or nontransfected MCA-102 tumor cells. The decreased tumorigenicity of E1A-expressing MCA-102 cells was dependent on an intact T cell and NK cell antitumor immune response. These studies provide the first animal model in which the immunogenicities of these viral oncoproteins have been directly compared and shown to influence primary tumor development. The possible biological implications of these findings are discussed.

RESULTS

Establishment of MCA-102 transfectants expressing high levels of HPV-E7 or Ad-E1A oncoproteins

MCA-102 cells were transfected with either pLSXN16E7 or pE1A-neo. Geneticin-resistant colonies were screened for HPV-16-E7 or Ad5-E1A expression by Western analysis. Levels of E7 oncoprotein expression in MCA-102-E7-CL1 and MCA-102-E7-CL2 were compared to those in SiHa and H4-E7. SiHa is an HPV16-transformed human cervical cancer line that expresses low levels of HPV16-E7, whereas H4-E7 is an HPV16-E7-transfected human fibrosarcoma cell line that expresses high levels of E7 (Routes and Ryan, 1995). As shown in Fig. 1, both MCA-102-E7-CL1 and MCA-102-E7-CL2 expressed considerably higher levels of the HPV16-E7 oncoprotein than SiHa, but slightly lower levels than H4-E7. Similarly, high levels of the Ad5-E1A oncoproteins were expressed in both MCA-102-E1A-CL1 and MCA-102-E1A-CL2 cells. The cell morphologies and *in vitro* doubling times of these E7- and E1A-expressing MCA-102 lines were indistinguishable from those of nontransfected, MCA-102 cells (data not shown).

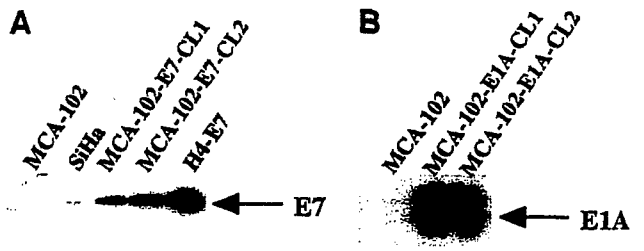

FIG. 1. (A) Expression of HPV16-E7 oncoprotein MCA-102-E7-CL1, MCA-102-E7-CL2, SiHa, and H4-E7 cell lines measured by Western analysis. (B) Expression of Ad5-E1A protein in MCA-102-E1A-CL1 and -CL2 cells measured by Western analysis.

Failure of E7-expressing MCA-102 cells to elicit a protective cellular immune response to primary tumor development

The effects of E7 oncogene expression on primary tumor development by MCA-102 cells was tested in mice with varying abilities to mount an antineoplastic cellular immune response (Fig. 2). If E7 expression were effective in increasing the immunogenicity of MCA-102 cells, it would be predicted that expression of this oncoprotein would reduce tumorigenicity in immunocompetent mice compared with that in T-cell-deficient, nude mice. This was not the observation. Only a few thousand parental MCA-102 cells were required to induce subcutaneous tumors in immunocompetent mice (TPD_{50} mean ± SEM = 3.3 ± 0.4 cells) (Fig. 2 and Table 1). Expression of high levels of E7 in two different transfected clones did not alter this level of tumorigenicity (TPD_{50} = 3.4 ± 0.3 and 2.8 ± 0.2). This failure of E7 expression to alter tumorigenicity was also observed with cells co-expressing HPV E6 (TPD_{50} = 3.0 ± 0.3). There was also no differential effect of E7 expression on MCA-102 tumor development in T-cell-deficient nude mice (Fig. 3). Both parental MCA-102 cells and E7-expressing cells were slightly more tumorigenic in nude than in immunocompetent mice, as indicated by the slightly lower TPD_{50} values of each cell type in nude mice (Fig. 3 vs Fig. 2). There were, however, no significant differences in the tumor-inducing capacities of E7-expressing cells compared with parental cells when compared in either nude (Fig. 3) or normal mice (Fig. 2). These data suggested that E7 expression does not induce an effective T-cell-dependent defense against primary tumor formation by MCA-102 cells.

MCA-102 cells are inherently nonimmunogenic in syngeneic C57/BL6 mice (Chen *et al.*, 1994; Mule *et al.*, 1987; Yang *et al.*, 1995). Therefore, it was possible that these cells possessed some trait that limited the immunogenic effects of any transfected viral oncoproteins. This possibility was tested in two ways. MCA-102 cells expressing E1A, rather than E7, were tested in the same tumorigenicity assays in immunocompetent mice (Fig. 2). In contrast to E7 expression, E1A expression caused a three- to four-log decrease in tumorigenicity of transfected MCA-102 cells, as indicated by the respective increases in the numbers of cells required to induce tumors in 50% of animals (TPD_{50} of E1A-positive cells, E1A-CL1 = 6.8 ± 0.4, E1A-C12 = 7.3 ± 0.3 cells vs TPD_{50} of parental cells = 3.3 ± 0.4 cells). This comparison of different MCA-102 clones transfected with different viral oncogenes could not exclude the possibility that clonal variation in the different transfectants contributed to the differences in tumorigenicity. Therefore, in a second type of experiment, an E7-transfected clone of MCA-102 cells was supertransfected with E1A and retested for sensi-

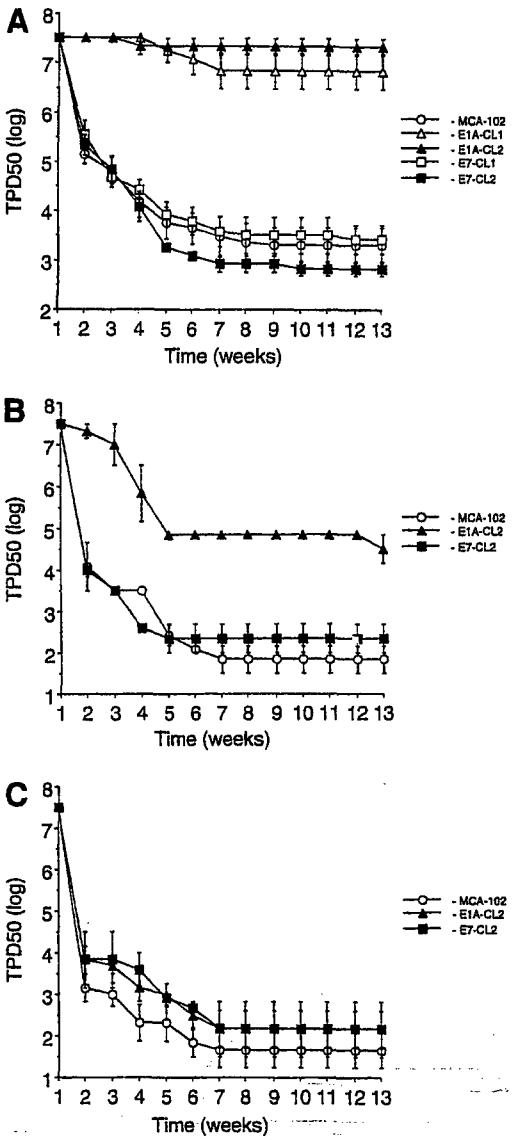

FIG. 2. Tumor induction studies in normal, nude, and CD3-ε-transgenic mice. Immunologically normal (A), nude (B), and CD3-ε-transgenic mice (C) were injected subcutaneously in the flank with log dilutions of MCA-102, MCA-102-E1A, and MCA-102-E7 cells and observed weekly for 12 weeks. Animals were sacrificed when tumors reached a mean diameter of 20 mm or at the end of a 12-week observation period. The calculated TPD_{50} value represents the $\log_{10}$ of the number of tumor cells required to produce tumors in 50% of the mice.

TABLE 1

Tumor-Inducing Capacity, Class I MHC Antigen Surface Expression, and NK Cell Killing of MCA-102 Lines

Cell line	TPD_{50} Normal mice	Class I MHC antigen surface expression[a] (%)	Sensitivity to NK cell lysis
MCA-102	3.3 ± 0.4	100	No
-E1A-CL1	6.8 ± 0.4	120 ± 5	Yes
-E1A-CL2	7.3 ± 0.3	400 ± 7	Yes
-E7-CL1	3.4 ± 0.3	200 ± 6	No
-E7-CL2	2.8 ± 0.2	200 ± 4	No
-E7/E6	3 ± 0.3	220 ± 3	No
-E1A/E7	6.7 ± 0.2	290 ± 5	Yes

[a] Surface class I MHC antigen levels were normalized to the levels expressed on MCA-102 cells.

tivity to rejection by immunocompetent mice (Table 1). These E7/E1A coexpressing cells exhibited the same three-log reduction in tumorigenicity in immunocompetent mice that was observed with cells expressing E1A alone. This eliminated clonal selection as an explanation for the observed differences in tumorigenic phenotypes of the cells and further excluded the possibility that there was some trait of E7-expressing cells that blocked expression of a rejection-susceptible phenotype. These data suggested that, in contrast to E1A expression, E7 expression was weakly immunogenic (or nonimmunogenic) in MCA-102 cells and failed to elicit a protective host defense against primary tumor formation.

E1A-expressing MCA-102 cells elicit both NK cell- and T-cell-specific responses to primary tumor challenge

The results of two types of animal experiments suggested that, in addition to a T-cell-dependent rejection response, the reduced tumorigenicity of E1A-expressing MCA-102 cells was also dependent on NK cell-mediated rejection. First, depletion of NK cells *in vivo* by pretreatment of normal mice with the anti-NK antibody PK136 (Koo *et al.*, 1986) increased the tumorigenicity of E1A-expressing cells, as evidenced by a 1.7-log reduction in the number of MCA-102-E1A cells required to produce tumors (TPD_{50} in NK cell-depleted mice = 4.8; TPD_{50} in untreated mice = 6.5). Second, E1A-expressing MCA-102 cells continued to be significantly less tumorigenic in T-cell-deficient nude mice than either parental MCA-102 or E7-expressing MCA-102 cells (TPD_{50} values, MCA-102-E1A = 4.5 vs MCA-102 = 1.8 and MCA-102-E7 = 2.3). Nude mice have normal or increased NK cell activity. These results indicated that E1A but not E7 expression sensitized MCA-102 cells to an NK cell-dependent rejection response that is discernible from CTL-dependent rejection.

It was also possible that T-cell-independent defenses other than NK cells contributed to the enhanced rejection of E1A-, but not E7-, expressing cells in nude mice (Cook *et al.*, 1982). To test this, tumor induction studies were done in CD3-ε-transgenic mice, which lack both NK cells and T cells (Fig. 2C). There were no significant differences in the tumor-forming capacities of MCA-102-E1A cells in these mice, compared with either parental MCA-102 cells or MCA-102-E7 cells. Therefore, the incremental deletion of the NK cell response in addition to the T cell response that resulted from using CD3-ε-transgenic mice, rather than nude mice, resulted in complete elimination of the host's ability to reject E1A-expressing cells. These results also showed that the reduced tumorigenicity of E1A-expressing MCA-102 cells in nude mice was NK cell-dependent and was not explained by some other, lymphocyte-independent rejection mechanism.

Inverse correlation between NK susceptibility of oncogene-expressing tumor cells tested *in vitro* and tumorigenicity in NK-competent animals

Next, we tested the susceptibilities of several MCA-102 lines expressing either E7 or E1A to NK cell killing to determine if patterns of lysis *in vitro* correlated with cell line susceptibility to protective NK and T cell responses *in vivo* (Fig. 3). Expression of E1A as a single oncogene induced MCA-102 cells to become highly susceptible to NK cell killing. In contrast, E7 expression resulted in no increase in NK susceptibility compared with nontransfected, MCA-102 cells. To exclude the possibility that these NK susceptibility patterns were specific to the cell clone, rather than the oncogene expressed, an E7-expressing clone of MCA-102 cells that had been supertransfected with E1A and a second clone that had been established by co-transfection with E7 and E6 were tested in NK cell assays. E1A expression converted the

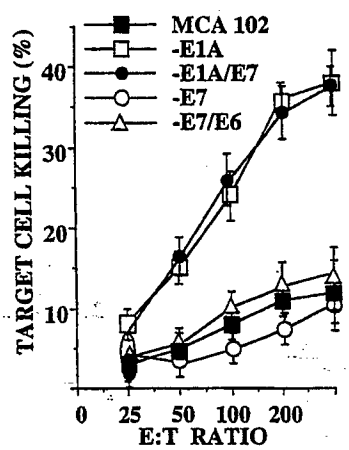

FIG. 3. Susceptibility of MCA-102 lines to NK cell lysis. NK lysis of MCA-102-E1A-CL1, MCA-102-E7-CL1, MCA-102-E1A/E7, and MCA-102-E7/E6 cells as measured by a 6-h. ^{51}Cr-release cytolysis assay. Results represent the means ± SEM of four separate experiments. Equivalent patterns and magnitude of NK cell killing were seen with MCA-102-E1A-CL2 and MCA-102-E7-CL2 cells (data not shown).

E7-expressing clone from an NK-resistant to an NK-susceptible cell. This result indicated that E7 expression did not select for an inherently NK-resistant clone. The failure of the E7/E6 coexpressing MCA-102 clone to exhibit NK susceptibility further indicated that the E7-related resistant phenotype observed with the single oncogene E7 transfectant was not a result of clonal selection.

NK resistance and tumorigenicity of oncogene-expressing MCA-102 cells do not correlate with the level of class I MHC antigen expression

NK cells express inhibitory receptors that recognize class I MHC antigens expressed on target cells (Lanier, 1998). In some experimental systems, cells that express high levels of class I antigens are resistant to NK cell killing, whereas cells that express low levels of class I are NK sensitive (Lanier, 1998). If this were the explanation for the oncogene-related NK and tumorigenic phenotypes observed in these studies, it would be predicted that cells in which E1A expression had induced high-level NK susceptibility would express very low levels of class I MHC antigens, relative to NK-resistant parental cells and E7-expressing cells. In contrast, it has also been proposed that the escape of Ad- or HPV-transformed cells from the T-cell-mediated cellular immune response occurs as a result of downregulation of cell surface expression of class I MHC antigens (Bernards *et al.*, 1983; Cromme *et al.*, 1994; Schrier *et al.*, 1983). If this were the case with the MCA-102 cells tested here, it would be predicted that the nonimmunogenic, E7-expressing MCA-102 cells tested here would express very high levels of class I antigen compared with the E1A-expressing cells. It was also possible that there was no correlation between class I MHC antigen expression and susceptibility to NK killing or to T-cell-mediated rejection, as had been reported from some studies of DNA virus-transformed rodent and human cells (Haddada *et al.*, 1986, 1988; Routes and Cook, 1995).

To examine these relationships using the oncogene-expressing MCA-102 cells studied here, surface class I MHC antigen expression was measured by FACS analysis (Table 1). The results showed that all oncogene-transfected clones of MCA-102 cells expressed higher levels of class I MHC antigen than did nontransfected, parental MCA-102 cells. More important for the purpose of the above considerations, there was no correlation between class I antigen expression and either tumorigenicity or NK susceptibility of the clones. Levels of K^b and D^b surface expression on all MCA-102 lines were also measured by flow cytometry and directly correlated with total class I antigen levels. Therefore, levels of surface class I MHC antigen expression did not explain either the NK phenotypes or the relative tumorigenicities of these oncogene-expressing cells.

DISCUSSION

Viral oncogene-induced cellular immortalization is a prerequisite for establishment of stable, neoplastic cell clones, but this immortalization step is only part of the explanation for tumorigenicity. Comparison studies of different Ad serotypes and other papovaviruses have shown that another important factor contributing to the differences in the tumorigenicities of cells immortalized by different viral oncogenes is the variable outcomes of interactions between oncogene-immortalized cells and components of the host cellular immune response (Lewis and Cook, 1984, 1985). For example, group A and C adenoviruses are equally competent to transform mammalian cells, but only group A Ad are oncogenic in immunocompetent rodents (Gallimore and Paraskeva, 1980). Studies using Ad5 (nontumorigenic)–Ad12 (highly tumorigenic) chimeric viruses showed that the differences in the oncogenicities of these viruses are regulated primarily by the functions of their E1A oncoproteins (Bernards *et al.*, 1982; Jelinek *et al.*, 1994; Telling and Williams, 1994). These E1A differences also determine whether Ad2/5- or Ad12-transformed cells elicit protective NK and T cell responses. Expression of Ad2/5, but not Ad12, E1A gene products sensitizes cells to lysis by NK cells (Cook and Lewis, 1984; Cook *et al.*, 1986). Similarly, Ad2/5-, but not Ad-12-, transformed cells elicit robust CTL responses (Bellgrau *et al.*, 1988; Pereira *et al.*, 1995). We speculate that a weak NK response to Ad12-transformed cells may contribute to the subsequent failure to generate a strong Ad12-E1A-CTL response *in vivo* (see below). Alternative explanations for a blunted CTL response include the capacity of Ad12-E1A gene products to transrepress the expression of class I MHC antigens and the TAP1 and TAP2 transporter genes (Bernards *et al.*, 1983; Schrier *et al.*, 1983), an activity not shared by Ad5-E1A gene products.

These observations suggest that, in addition to E1A-induced cellular immortalization, E1A-induced cellular traits that modulate the cellular immune response to oncogene-expressing cells are an important determinant of tumor progression or rejection. These studies may be relevant for discerning the reasons for the dissimilar oncogenicities of HPV and Ad in humans. In a manner analogous to that of differences between highly oncogenic Ad12 and nononcogenic Ad2/5-E1A genes, the dissimilarities in oncogene function between HPV16-E7 and Ad5-E1A may influence the tumorigenicities of Ad- and HPV-transformed cells in humans.

The studies presented here are consistent with this hypothesis and suggest that one reason for the tumorigenicity of E7 oncogene-expressing tumor cells is the failure of these cells to initiate an effective antineoplastic cellular immune response. For example, E1A expression caused a marked reduction of the tumorigenicity of MCA-102 cells in immunocompetent mice and a significant

reduction of MCA-102 tumorigenicity in T-cell-deficient, NK-competent nude mice. The observation that E1A had no effect on tumor development in CD3-ε-transgenic mice that lacked both NK cell and T cell defenses indicates that this E1A-induced reduction in tumorigenicity was caused by the combined effects of T cell and NK cell defenses (Figs. 2 and 3 and Table 1). In contrast, E7 expression failed to induce any detectable tumor rejection response in NK- and T-cell-competent mice.

The lack of a protective T-cell-dependent response against E7-expressing MCA-102 cells (Fig. 2) cannot be explained by the lack of antigenicity in the E7 protein itself in the H-2^b background of the C57/BL6 mice used in these experiments. There are several reports showing that E7 is highly antigenic in these mice. For example, E7 peptide immunization of C57/BL6 mice induces E7-specific CTL activity (Feltkamp *et al.,* 1993; Sadovnikova *et al.,* 1993). Furthermore, such CTL-inducing vaccination strategies protect mice from tumor development by E7-expressing tumor cell lines (Feltkamp *et al.,* 1995; Ossevoort *et al.,* 1995). This same pattern of viral oncogene-induced protection following preimmunization but absence of protection during primary tumor development has been reported for several other oncogenic DNA tumor viruses, including SV40 expressed in hamster cells and Ad12 expressed in hamster and mouse cells (Levine *et al.,* 1984; Lewis and Cook, 1984, 1985). It is clear, therefore, that the antigenicity of an oncoprotein does not guarantee its ability to induce tumor cell rejection during primary tumor formation. In fact, this is usually not the case.

This failure of E7 expression to induce primary tumor rejection is common to both the human host faced with HPV-transformed cervical carcinoma cells and the C57/BL6 mouse challenged with E7-expressing MCA-102 cells. One possible explanation for this phenomenon that would be consistent with the data presented in this report and with our previous studies of HPV-transformed human cells is that there is a requirement for an "initiating interaction" between oncogene-expressing tumor cells and NK cells that is required for effective generation of an oncoprotein-specific CTL response. There is precedent for such an accessory role for NK cells in the generation of a CTL response (Kos and Engleman, 1996). For example, it has been reported that depletion of NK cells reduces generation of syngeneic tumor-specific, virus-specific, and allospecific CTL (Kos and Engleman, 1996; Kurosawa *et al.,* 1995; Suzuki *et al.,* 1985). Our data and those in previous reports also show that certain viral oncogenes fail to sensitize the cells they immortalize to NK killing and that these NK-insensitive oncogene-expressing cells do not induce protective, oncoprotein-specific anti-tumor responses. There are several examples of this correlation between the ability of a DNA viral oncogene to induce cellular sensitization to NK killing and the tumor-inducing capacity of the oncogene-expressing cell in the immunocompetent animal. This correlation has been reported for hamster and mouse cells expressing Ad2/5-E1A (NK susceptible and nontumorigenic), hamster and mouse cells expressing Ad12-E1A (NK resistant and tumorigenic), SV40 T antigen-expressing hamster cells (NK resistant and tumorigenic), SV40 T antigen-expressing mouse and rat cells (NK sensitive and nontumorigenic), polyoma T antigen-expressing hamster cells (NK resistant and tumorigenic), and human cells expressing HPV16- or HPV18-E7 and -E6 (NK resistant and tumorigenic) (Cook *et al.,* 1980, 1982; Cook and Lewis, 1984; Fresa *et al.,* 1987; Raska and Gallimore, 1982; Routes and Ryan, 1995; Sawada *et al.,* 1985). The data reported here extend this correlation to E7-expressing MCA-102 cells (NK resistant and tumorigenic) and suggest that this same pattern of resistance for HPV16- or HPV-E7-expressing human tumor cells may be relevant *in vivo.* Furthermore, neither the NK sensitivity nor tumorigenicities of the different MCA-102 lines were related to class I MHC antigen expression. These data are in agreement with other studies relating NK sensitivity and either tumorigenicity or class I expression on Ad-transformed or E1A-transfected rodent and human cell lines (Haddada *et al.,* 1986, 1988; Routes and Cook, 1995). Studies are ongoing to determine if the expression of nonclassical class I antigens influences the NK sensitivities of E1A- and E7-expressing murine and human tumor cell lines.

The inability of E7-expressing MCA-102 cells to induce protective T-cell-dependent responses in immunocompetent mice is also consistent with observations of HPV-transformed cells in humans and other types of E7-transfected tumor cells in mice. For example, E7- or E6-specific CTL are inefficiently generated in women with HPV-induced cervical cancer (Borysiewicz *et al.,* 1996; Evans *et al.,* 1996; Ressing *et al.,* 1996). Similarly, injection of HPV16-E7-transfected tumor cells that are not co-transfected with the co-stimulatory molecule B7.1 fail to induce E7-specific CTL in mice (Chen *et al.,* 1992). Thus, our findings are consistent with the hypothesis that the E7 and E6 oncoproteins are ignored by the immune system despite their antigenicity, a state referred to as immunological ignorance (Melero *et al.,* 1997).

HPV infect only human keratinocytes with complete viral replication linked to the differentiation of the infected cell. HPV gene products that help circumvent the host immune response to HPV infection may exist. In addition, the urogenital location of HPV-induced malignancies as well as differences in the replicative cycle and the unique cell tropism of HPV may contribute to the dissimilar oncogenicities of HPV and Ad. However, we believe that these are not the only factors leading to the dissimilar oncogenicities of Ad and HPV. Ad are ubiquitous human pathogens that cause persistant infections with asymptomatic fecal excretion for months to years following the initial infection (Fox *et al.,* 1969). Ad infect

epithelial (including keratinocytes), fibroblastic, and lymphoidal cells. Ad can cause both asymptomatic and symptomatic urogenital tract infections (Mufson *et al.*, 1973). Like HPV-E7/E6, Ad-E1A/E1B are competent to completely transform both epithelial and fibroblastic cells *in vitro*. Furthermore, Ad-transformed human cells are tumorigenic in immunodeficient animals (Chang *et al.*, 1990). Therefore, the fact that Ad can infect and potentially transform cells at virtually any anatomic location, including locations in which HPV replicate, precludes the possibility that the cell tropism and anatomic site of HPV-induced malignancy is the only explanation for the dissimilar oncogenicities of Ad and HPV. Finally, although there may be HPV proteins produced that circumvent cellular immunity to HPV infection, these proteins likely would not affect the cellular immune response following viral transformation. HPV-transformed cells are virion free and no further viral replication is possible (Galloway and McDougall, 1989). Only two viral proteins are consistently expressed in HPV-transformed cells, the E7 and E6 oncoproteins.

In summary, the data reported here show that the dissimilar immunogenicities of the E1A and E7 oncoproteins can influence primary tumor development. We postulate that differences similar to those observed in this mouse model might also exist in the cellular immune responses to human cells expressing Ad5-E1A or HPV16-E7. This hypothesis predicts that, following Ad5 transformation of human cells, E1A expression would elicit a robust NK cell and T cell response that results in destruction of these cells *in vivo*. In contrast, following HPV16 transformation, E7 expression would fail to elicit such an immune response and would, therefore, persist to allow cellular transformation, subsequent cellular mutations, and tumor progression. These observations provide a basis for future studies contrasting E7 and E1A to test cellular mechanisms that explain the failure of E7 to sensitize tumor cells to NK killing and to induce T-cell-dependent tumor rejection by immunocompetent animals.

MATERIALS AND METHODS

Cell lines

The methylcholanthrene-induced sarcoma cell line MCA-102 was provided by Dr. Nicholas Restifo (National Institutes of Health, Bethesda, MD) (Mule *et al.*, 1987). H4-E7 cells are HT1080-derived, human fibrosarcoma cells expressing high levels of the HPV16-E7 oncoprotein (Routes and Ryan, 1995). MCA-102 cells expressing Ad5-E1A or HPV16-E7 were derived from clones selected in G418 following transfection with pLSXN16E7 or pE1A-neo, which code for G418 resistance and HPV16-E7 or Ad5-E1A, respectively. G418-resistant colonies were expanded and screened for the expression of Ad5-E1A or HPV16-E7 by Western analysis. pLSXN16E7 was provided by Denise Galloway (Fred Hutchinson Cancer Research Center, Seattle, WA) (Halbert *et al.*, 1991). pE1A-neo was provided by Elizabeth Moran (Temple University, Philadelphia, PA) (Ruley *et al.*, 1985). Two independently derived clones of MCA-102 cells expressing high levels of either Ad5-E1A (MCA-102-E1A-CL1, MCA-102-E1A-CL2) or HPV16-E7 (MCA-102-E7-CL1, MCA-102-E7-CL2) were used for all studies. The MCA-102 cell lines were maintained in DMEM supplemented with antibiotics, 15 mM glucose, and 5% FCS. Cell lines were periodically tested for contamination with mycoplasma using the Mycotec assay (Bethesda Research Labs, Bethesda, MD) and were negative.

NK cell cytolysis assays

NK cytolysis assays were performed as described using spleen cells from athymic nude C57/B6 mice as the source of NK cells and target cells labeled with [^{51}Cr] (100 mCi/ml for 1 h; 1 Ci = 37 gBq) (Routes and Cook, 1995). The results shown represent the means ± SEM of at least four separate experiments. The mean percentage spontaneous release from all types of target cells was less than 20%.

Western analysis

For quantitation of E1A proteins, 60-mm plates of MCA-102-E1A-CL1 or MCA-102-E1A-CL2 cells were lysed in RIPA buffer (1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.4, 150 mM NaCl), and protein concentrations of RIPA supernatants were determined by the BCA protein assay (Pierce, Rockford, IL). An equal amount of protein from each cell lysate was separated on 10% SDS–PAGE polyacrylamide gels and electrophoretically transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 5% nonfat milk solution and incubated with the anti-E1A monoclonal antibody, M73, supplied by E. Harlow (Massachusetts General Hospital, Charleston, MA) for 1 h (Harlow *et al.*, 1985). Following several washes with PBST (PBS with 0.05% Triton X-100), the membranes were incubated for 1 h with rabbit anti-mouse antibody (Cappel, Durnham, NC) and washed extensively with PBST. The E1A protein was then visualized per the manufacturer's instructions using the Renaissance Chemiluminescence Kit (DuPont–NEN, Boston, MA).

For quantitation of E7 proteins, 100-mm plates of H4-E7, SiHa, or MCA-102-E7-CL1 and CL2 cells were lysed with the E7 lysis buffer (20 mM Tris, ph-7.5, 150 mM NaCl, 0.5% SDS, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, 1% aprotinin, and 1 μM PMSF). Protein concentrations from each lysate were determined and equal amounts of lysate were immunoprecipitated using Protein A Sepharose CL-4B beads (Amersham Pharmacia Biotech, Uppsula, Sweden) and the E7 monoclonal antibody ED17 (Santa Cruz Biotechnology, Santa Cruz, CA). Immunopre-

cipitated proteins were resolved on 16% SDS–PAGE and transferred to PVDF membrane. With the exception of the use of the monoclonal E7 antibody, the remainder of the Western analysis was identical to the procedure used for the detection of the E1A proteins.

Tumor induction studies

CD3-ε-transgenic mice and congenitally athymic nude and normal C57/BL6 mice were obtained from Jackson Laboratories. CD3-ε-transgenic mice do not express either T cells or NK cells (Wang *et al.*, 1994). Quantitative tumor induction studies were performed as previously described (Walker *et al.*, 1991). Briefly, mice (three animals per cell dose for normal and athymic nude mice, two animals per dilution for CD3-ε-transgenic mice) were injected subcutaneously with serial log concentrations of cells and observed weekly for tumor development for 12 weeks. Animals were sacrificed when tumors reached a mean diameter of 20 mm or at the end of the 12-week observation period. Tumor cells from animals injected with either MCA-102-E1A or MCA-102-E7 cells were tested for E1A or E7 expression. TPD_{50} (log_{10} of the number of tumor cells required to produce tumors in 50% of the mice) were calculated by the method of Karber (1931).

Measurement of class I MHC antigen levels

M1/42.3.98, a monoclonal antibody that is panreactive to all murine class I antigens, was obtained from the American Tissue Culture Collection. The MCA-102 lines were stained with M1/42.3.98, and 5000 cells were analyzed on an Epics C flow cytometer as described (Routes and Cook, 1990). The levels of surface class I antigens on the E1A- or E7-transfected cell lines were normalized to parental MCA-102 cells using linearized values of log mean fluorescence. The results in the text represent the means ± SEM of at least three experiments. Levels of K^b and D^b surface expression on all MCA-102 lines were also measured by flow cytometry and directly correlated with total class I antigen levels.

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E1A ONCOGENE SENSITIZATION OF BREAST CANCER CELLS TO APOPTOTIC INJURY

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Human breast cancer cells are often resistant to injuries inflicted by immune defenses and chemotherapeutic agents. A means to sensitize such resistant cells to these injuries might be useful in designing new strategies for treatment. We have reported that expression of the adenoviral E1A oncogene in both rodent and human cells sensitizes them to diverse apoptotic injuries. The purpose of this study was to test the hypothesis that E1A expression in human breast cancer cells will render them sensitive to both immune-mediated and chemotherapeutic drug-induced apoptosis.

Human ductal adenocarcinoma breast cancer cells (MDA-MB-435S) were tested for sensitivity to of pro-apoptotic injuries before and after stable expression of E1A proteins. E1A-negative cells were resistant to most types of injuries. E1A expression converted these apoptosis-resistant cells into cells that were sensitive to lysis following apoptosis induced by human natural killer cells, the TNF-related apoptosis-inducing ligand (TRAIL), and the chemotherapeutic agent, etoposide. The apoptotic nature of cell death following exposure to these injuries was assessed using studies of nuclear morphology, DNA laddering, quantitation of DNA degradation and chromium release.

p53 gene mutations are common in human malignancies, including breast cancer. p53 expression can also be important in injury-induced apoptosis. Both rodent and human cells were tested for the p53-dependence of apoptosis triggered by immune-mediated and chemically-mediated injury. Immune-mediated injury of E1A-expressing cells occurred independently of p53 expression in both rodent and human cells. The p53-dependence of chemically-induced apoptosis was cell type specific. In rodent cells, p53 expression was required for an apoptotic response to most types of chemical injuries. In contrast, human cells lacking p53 expression, could be highly sensitive to a variety of chemical injuries.

The data indicate that E1A expression sensitizes human tumor cells, including breast cancer cells, to pro-apoptotic injuries triggered by both immune and chemically-induced mechanisms. The results also suggest that p53 gene mutations are insufficient to block this E1A-induced phenotypic change in many types of tumor cells.

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E1A-Induced Repression of the NF-kappa B Defense Against Apoptosis through a p300-Binding-Independent Mechanism

Jim Cook, Kelley Colvin, Barbara Routes, Tom Walker, Jay Radke and Wei-Yun Zheng
University of Illinois at Chicago

E1A expression sensitizes mammalian cells to apoptosis triggered by p53-independent immunological (e.g., cytolytic lymphocyte and TNF) and p53-dependent chemical and physical injuries. The possible mechanisms of this E1A activity include enhancement of the apoptotic cascade and repression of antiapoptotic defenses. One cellular defense against TNF-induced apoptosis involves activation of NF-kappa B (NF-kB)-dependent responses. E1A represses NF-kB-dependent transcription, but the cause and effect relationship between this effect and sensitization to apoptosis is unclear.

Stable or transient expression of Ad5 E1A sensitized NIH-3T3 cells to TNF-induced apoptosis and repressed TNF-induced kB-luciferase activity in an E1A-dose-related manner. E1A proteins encoded by both 12S and 13S mRNAs had the same repressive effect. Overexpression of the NF-kB p65/RelA subunit reversed E1A repression of transcription in a p65-dose-dependent manner and rescued E1A-positive cells from TNF-induced apoptosis, suggesting that these E1A effects are linked and p65/RelA-related.

Studies were done to define the NF-kB activation step that is blocked by E1A. NF-kB p65/RelA and p50/KBF1 subunit expression, TNF-induced I-kappa B turnover, NF-kB subunit nuclear translocation, heterodimerization, and binding of p65/p50 to kB oligomers were unaffected by E1A, suggesting that E1A repression of NF-kB-dependent transcription occurs at a stage after NF-kB binding to the enhancer. The most likely mechanism of this anti-NF-kB activity was through E1A binding to p300, a known coactivator of NF-kB-dependent transcription. Studies using p300-nonbinding E1A mutant proteins showed that, albeit not quite as effective as wild type E1A proteins, these mutants still repressed TNF-induced, NF-kB-dependent transcription in a dose-dependent manner and sensitized cells to TNF. The data suggest that E1A-induced apoptosis sensitization that occurs in response to p53-independent injury initiation pathways is caused in part by E1A repression of the cellular NF-kB defense through binding-independent, as well as binding-dependent, effects of E1A on p300 coactivator function.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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